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Determination of Total and Free Phenytoin in Serum by Non-Isotopic Immunoassays and Gas Chromatography[1])

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Summary: Four different homogeneous non-isotopic immunoassays for the determination of total phenytoin in serum were evaluated and compared with a gaschromatographic method (GC) described by *W. R. Külpmann & M. Oellerich* ((1981), J. Clin. Chem. Clin. Biochem. *19*, 249–258): enzyme multiplied immunoassay technique (EMIT), fluorescence polarization immunoassay (FPIA), nephelometric inhibition immunoassay (NIIA) and substrate labeled fluorescent immunoassay (SLFIA).

The between-days coefficients of variation in the medium therapeutic range were 4.0% (n = 29) with EMIT, 4.6% (n = 15) with FPIA, 7.8% (n = 10) with NIIA, 2.8% (n = 12) with SLFIA and 5.7% (n = 15) with GC. The recovery in spiked serum samples (phenytoin concentration: 11.9–99.1 µmol/l) was 98–101% with EMIT, 97–107% with FPIA, 102–110% with NIIA, 94–97% with SLFIA and 97–104% with GC.

All of the tested immunoassays and GC yielded comparable results. The NIIA showed a somewhat lower correlation. In samples from an uraemic patient, however, great deviations from GC values were obtained with EMIT (bias: +22 to +85%) NIIA (+68 to +114%) and SLFIA (+48 to +52%). Only the results of FPIA were in good agreement with those of GC (bias: +1 to −7%).

All the immunoassays showed a cross-reaction with 5-(4-hydroxyphenyl)-5-phenylhydantoin, which was most expressed with SLFIA and NIIA. The detectability of the immunoassays was adequate to allow precise measurements within the therapeutic range.

After ultrafiltration of the serum, free phenytoin was measured by EMIT, FPIA and capillary gas chromatography. The immunoassays and capillary GC yielded comparable results, though a small positive bias between EMIT and FPIA was noticeable. In a sample from an uraemic patient, however, the results of EMIT differed by +109% from the GC value, whereas FPIA showed a bias of only −12%.

Bestimmung von Gesamt- und freiem Phenytoin im Serum mit radioaktivitätsfreien Immunoassays und Gaschromatographie[1])

Zusammenfassung: Vier verschiedene homogene, radioaktivitätsfreie Immunoassays zur Bestimmung von Gesamt-Phenytoin im Serum wurden evaluiert und mit einer von *W. R. Külpmann & M. Oellerich* ((1981), J. Clin. Chem. Clin. Biochem. *19*, 249–258) beschriebenen gaschromatographischen Methode (GC) verglichen: Enzyme multiplied immunoassay technique (EMIT), fluorescence polarization immunoassay (FPIA), nephelometric inhibition immunoassay (NIIA) und substrate labeled fluorescent immunoassay (SLFIA). Der Variationskoeffizient für die Präzision von Tag zu Tag betrug im mittleren therapeutischen Bereich: 4.0% (n

[1]) Parts of this study have been reported at the Gemeinsame Jahrestagung Klinische Chemie der Deutschen Gesellschaft für Klinische Chemie gemeinsam mit der Österreichischen Gesellschaft für Klinische Chemie und der Österreichischen Gesellschaft für Laboratoriumsmedizin, Stuttgart, 22.–24. September, 1982.

= 29) für EMIT, 4,6% (n = 15) für FPIA, 7,8% (n = 10) für NIIA, 2,8% (n = 12) für SLFIA und 5,7% (n = 15) für GC. Die Wiederfindung von Phenytoin in aufgestockten Proben im Bereich zwischen 11,9–99,1 µmol/l schwankte zwischen 98–101% bei EMIT, 97–107% bei FPIA, 102–110% bei NIIA, 94–97% bei SLFIA und 97–104% bei GC. Die Ergebnisse der Phenytoinbestimmung, die bei Patientenproben mit den verschiedenen Methoden durchgeführt wurden, wurden mit denen der GC verglichen. Insgesamt ergab sich eine zufriedenstellende Korrelation, wenngleich die mittels NIIA erhaltenen Werte etwas weniger gut übereinstimmten. In Proben von einem urämischen Patienten unter Phenytointherapie wurden jedoch große Abweichungen zu den gaschromatographisch ermittelten Werten gefunden: EMIT: +22 bis +85%, NIIA: +68 bis +114%, SLFIA: +48 bis +52%. Lediglich die mittels FPIA gewonnenen Ergebnisse stimmten gut mit denen der GC überein (Abweichungen zwischen +1 und −7%). Alle Immunoassays zeigten eine Kreuzreaktion mit 5-(4-Hydroxyphenyl)-5-phenylhydantoin, die am stärksten ausgeprägt war bei SLFIA und NIIA. Innerhalb des therapeutischen Bereiches erlaubten alle Immunoassays präzise Bestimmungen.

Freies Phenytoin wurde mittels Ultrafiltration abgetrennt und mit EMIT, FPIA und Kapillargaschromatographie bestimmt. Die Immunoassays und die Kapillar-GC ergaben vergleichbare Ergebnisse, wenn auch die Werte von EMIT etwas höher lagen als die von FPIA. Die Bestimmung des freien Phenytoin bei einem urämischen Patienten unter Phenytointherapie ergab jedoch ein Ergebnis mittels EMIT, das 109% höher lag als der gaschromatographische Vergleichswert, während die entsprechenden Werte von GC und FPIA nur um 12% voneinander abwichen.

Introduction

Therapeutic drug monitoring of total phenytoin in epileptic patients has been widely used in the past few years. Most of the determinations were performed by GC and EMIT. Recently several new immunological methods for the determination of phenytoin have become commercially available. In this study three of these newer immunological methods: FPIA, NIIA and SLFIA were evaluated. The results from these immunoassays were compared with those from GC. Serum samples of an uraemic patient were included, because the accumulation of drug metabolites or endogenous compounds might be expected to cause interferences that are not usually encountered. In addition, the reliability of the determination of free phenytoin by EMIT and FPIA was tested.

Material and Methods

Determination of total phenytoin in serum

Immunoassays

1. Enzyme multiplied immunoassay technique (EMIT)

Reagents were purchased from E. Merck, Darmstadt. The determinations were performed according to the manufacturer's instructions by use of the EMIT-AutoLab 5000 System (Syva-Merck, Darmstadt), or according to an adaptation of the orginal procedure to the Eppendorf Analyzer 5010 and Eppendorf ACP 5040 (Eppendorf, Hamburg) (2).

2. Fluorescence polarization immunoassay (FPIA)

Reagents were obtained from Abbott (Wiesbaden). The determinations were performed according to the manufacturer's intructions by use of the TDX Fluorescence Polarization Immunoassay System (Abbott, Wiesbaden) (3, 4, 5). The calibration curve once established for a reagent lot was checked in the subsequent runs by one standard and accepted, if the deviation from the target value did not exceed ± 5%. Usually recalibration was necessary after 1 month.

3. Nephelometric inhibition immunoassay (NIIA)

Reagents were supplied by Beckman (München). The determinations were performed according to the manufacturer's instructions by use of the Auto ICS Immunochemistry System (Beckman, München) (6, 7). For each run a calibration curve was prepared.

4. Substrate labeled fluorescent immunoassay (SLFIA)

Reagents were obtained from Miles (Miles-Ames Division, Frankfurt). The determinations were performed according to the manufacturer's instructions by use of the Ames Fluorostat (Miles-Ames Division, Frankfurt) (8, 9. 10). The calibration curve could be used for at least 2 weeks for the same reagent lot. The calibration curve was checked and corrected in each run by the highest calibration standard, if the deviation from the target value did not exceed 6%. Otherwise recalibration was necessary.

Gaschromatographic method

Phenytoin was determined by gas chromatography according to l. c. (1) by use of a Hewlett Packard 5880 A gas chromatograph (Hewlett Packard, Frankfurt).

Determination of free phenytoin

Immunoassays

Free phenytoin was separated by ultrafiltration using EMIT Free Level Filters I (E. Merck, Darmstadt) and determined by EMIT and FPIA according to the manufacturer's instructions.

Gaschromatographic method

Extrelut-1-columns (E. Merck, Darmstadt) were rinsed with 6 ml dichloromethane and dried by suction prior to use. Ultrafiltrates

(200 µl) (see above) were spiked with 100 µl internal standard (5-(4-methylphenyl)-5-phenylhydantoin; 10 mg/l) and applied to an Extrelut-1-column. After 10 min, phenytoin was eluted by 6 ml dichloromethane. The eluate was evaporated and the dry residue dissolved in 100 µl ethyl acetate for the gas chromatographic analysis. Gas chromatography was performed by use of a fused silica capillary column (length: 25 m; internal diameter: 0.31 mm) coated with cross-linked 5% phenylmethyl silicone (Hewlett-Packard, Frankfurt).

Inlet: Cool on column.

Oven temperature:
Initial value: 50 °C, initial time: 0.5 min
programme rate: 30 °C/min
final value 1: 220 °C, final time 1: 0.5 min
programme rate 2: 15 °C/min
final value 2: 260 °C, final time 2: 3.0 min

Detector: N-FID, 300 °C

Syringe: 10 µl capacity, fused silica needle 11 cm (Hamilton, Darmstadt); injected volume: 2 µl.

Control sera

EMIT antiepileptic drug control was purchased from E. Merck, Darmstadt).

ORTHO bi-level assayed anticonvulsant/antiasthmatic control set I or II were obtained from Ortho Diagnostics (Heidelberg).

Results

Precision

The between-days precision of the immunoassays is shown in table 1. The coefficients of variation for "EMIT antiepileptic drug control" calculated from determinations performed in duplicate were: SLFIA: 2.8%; EMIT: 4.0%; NIIA: 7.8%, and calculated from single determinations: FPIA: 4.6%.

Measuring range

In order to estimate the detectability of the methods the precision in the series at low concentrations was determined (tab. 2). All procedures allow precise measurements below the therapeutic range; SLFIA and NIIA, however, were less sensitive than the other assays. At a phenytoin concentration of 2.0 µmol/l the coefficient of variation was 15.9% with EMIT and 7.4% with FPIA.

Accuracy

Recovery

The recovery of phenytoin by the different immunoassays and by GC is presented in table 3. Phenytoin was added to pooled drug-free human serum. The recovery within the therapeutic range varied between 94 and 110%.

Tab. 1. Between-days precision of various immunoassays and GC for determination of phenytoin in serum.

Control serum	Target value (µmol/l)	EMIT[1] $\bar{x}$[3] (µmol/l)	EMIT[1] CV[3] (%)	EMIT[1] n[3]	FPIA $\bar{x}$[4] (µmol/l)	FPIA CV[4] (%)	FPIA n[4]	NIIA $\bar{x}$[3] (µmol/l)	NIIA CV[3] (%)	NIIA n[3]	SLFIA $\bar{x}$[3] (µmol/l)	SLFIA CV[3] (%)	SLFIA n[3]	GC[2] $\bar{x}$[4] (µmol/l)	GC[2] CV[4] (%)	GC[2] n[4]
EMIT antiepileptic drug control	59.5	59.5	4.0	29	57.1	4.6	15	61.8	7.8	10	60.6	2.8	12	59.6	5.7	15
ORTHO control I[5]	27.0	28.1	7.2	10	28.1	4.9	15	–	–	–	–	–	–	–	–	–
ORTHO control II[5]	74.5	76.5	5.3	8	–	–	–	–	–	–	–	–	–	–	–	–

[1] EMIT by the Eppendorf ACP 5040.
[2] GC according to l. c. (1).
[3] $\bar{x}$: Mean value, CV: Coefficient of variation calculated from determinations performed in duplicate, n: number of days.
[4] $\bar{x}$: Mean value, CV: Coefficient of variation calculated from single determinations during n days.
[5] ORTHO bi-level assayed anticonvulsant/antiasthmatic control set I resp. II.

Tab. 2. Recovery and precision at low concentrations of various immunoassays and GC for determination of phenytoin in serum.

Method	Spiked value (μmol/l)	Number of determinations	Mean $\bar{x}$ (μmol/l)	3 s[3]) (μmol/l)	CV (%)
EMIT[1])	9.9	10	9.8	0.9	3.2
	2.0	9	1.7	0.8	15.9
FPIA	9.9	10	8.9	0.9	3.4
	2.0	10	2.1	0.5	7.4
NIIA	9.9	8	9.0	1.3	4.8
SLFIA	9.9	10	7.2	2.4	11.2
GC[2])	4.0	10	3.7	1.2	10.6

[1]) EMIT by use of the AutoLab 5000 System.
[2]) GC according to l. c. (1).
[3]) s: Standard deviation.

Tab. 3. Recovery of phenytoin by various immunoassays and GC in spiked serum samples.

Phenytoin (μmol/l)	EMIT[1]) (%)[3])	n[3])	FPIA (%)[3])	n[3])	NIIA (%)[3])	n[3])	SLFIA (%)[3])	n[3])	GC[2]) (%)[3])	n[3])
9.9	–	–	110	5	91	4	84	5	104	5
11.9	98	6	97	2	–	–	–	–	–	–
19.8	–	–	–	–	110	4	94	5	104	5
29.7	101	6	107	5	–	–	–	–	–	–
39.6	–	–	–	–	103	4	97	5	100	5
59.5	100	6	105	5	–	–	–	–	–	–
79.3	–	–	–	–	102	4	97	5	97	5
99.1	99	6	–	–	–	–	–	–	–	–
118.9	–	–	102	5	100	4	101	5	98	5
158.5	–	–	95	3	101	4	–	–	–	–

[1]) EMIT by the Eppendorf ACP 5040.
[2]) GC according to l. c. (1).
[3]) Recovery (%) calculated from n single determinations.

Comparison of methods

Values obtained from analyses of patients' specimens by EMIT, FPIA, NIIA and SLFIA were compared with those from GC (tab. 4).

EMIT: A proportional error of 4% and a negligible constant error was obtained; the mean values differed by about ± 4%.

FPIA: A proportional error of 6% and a small constant error was observed; the difference between the mean values, however, was not significant.

NIIA: The proportional error was 10%, the intercept 2.5 μmol/l; the standard error of the residuals (8.6 μmol/l) was caused by the lower precision of this method.

SLFIA: In the absence of a proportional error the intercept was minimal and the mean values were almost identical.

On the whole it may be concluded that – though differences between some immunoassays and GC were obtained – the bias between the results would not be of clinical relevance for this group of patients.

Furthermore samples from an uraemic patient treated with phenytoin were analysed. Clinical chemical analyses in samples from this patient yielded the following results (reference values in parentheses):

creatinine 988 μmol/l (≤ 115 μmol/l),
urea 24.6 mmol/l (3.3–6.7 mmol/l),
uric acid 357 μmol/l (200–420 μmol/l),
cholesterol 5.4 mmol/l (≤ 7.2 mmol/l),
triglycerides 2.02 mmol/l (≤ 1.80 mmol/l),
protein 65 g/l (65–80 g/l),
albumin 40 g/l (37–51 g/l),
alanine aminotransferase 10 U/l (≤ 22 U/l),
aspartate aminotransferase 8 U/l (≤ 18 U/l),
alkaline phosphatase 138 U/l (76–190 U/l),
γ-glutamyltransferase 37 U/l (≤ 28 U/l),

Tab. 4. Comparison of the results obtained by various immunoassays and GC for determination of phenytoin in samples from patients treated with the drug.

Methods (y)	vs.	(x)	n[1])	Standardized principal component analysis Slope[9])	Intercept (μmol/l)	$s_{y \cdot x}$[2]) (μmol/l)	$\bar{y}$[3]) (μmol/l)	$\bar{x}$[4]) (μmol/l)	t[5])	Correlation coefficient r
EMIT[6])	vs.	GC[7])	42	0.96**	−0.4	2.3	44.0	46.0	3.94*	0.99
EMIT[8])	vs.	GC[7])	39	1.04**	−0.2	4.0	46.2	44.6	−1.70*	0.96
FPIA	vs.	GC[7])	42	0.94**	+1.4	3.3	44.8	46.0	1.66	0.98
NIIA	vs.	GC[7])	50	1.10**	−2.5	8.6	46.8	44.4	−1.38	0.92
SLFIA	vs.	GC[7])	42	1.00**	+0.1	3.5	46.4	46.0	−0.52	0.98

[1]) n: Number of contributing pairs of results.
[2]) $s_{y \cdot x}$: Standard error of residues.
[3]) $\bar{y}$: Mean value of method y.
[4]) $\bar{x}$: Mean value of method x.
[5]) t: t-value (paired t-test); *: significance of the bias $\bar{x}-\bar{y}$ ($p \leq 0.05$).
[6]) Determinations by EMIT adapted to an Eppendorf ACP 5040.
[7]) GC: Gas chromatographic determination according to l.c. (1).
[8]) Orginal procedure performed by use of an AutoLab 5000 System.
[9]) **: Not significant deviation from identity (t-test; $p \leq 0.05$).

creatine kinase 37 U/l (≤ 70 U/l),
potassium 5.6 mmol/l (3.6–5.4 mmol/l),
sodium 135 mmol/l (132–155 mmol/l),
calcium 1.99 mmol/l (2.15–2.60 mmol/l),
phosphate, inorganic 3.00 mmol/l (0.83–1.67 mmol/l).

The phenytoin concentrations determined in samples of this patient by all of the immunoassays except FPIA were much higher than those obtained by GC (tab. 5). EMIT results differed by 22–85%, NIIA by 68–114% and SLFIA constantly by about 50%, whereas FPIA differed by only +1 to −7%. The erroneous results are probably not due to endogenous compounds: When serum of an uraemic patient (creatine: 1088 μmol/l serum) was spiked with phenytoin (final concentration: 59.5 μmol/l) almost identical results were obtained by EMIT and FPIA (55.9 resp. 56.7 μmol/l).

Free phenytoin

Free phenytoin was determined by EMIT, FPIA and capillary GC. Free phenytoin was obtained by ultrafiltration without loss by adsorption (11). When the results of these methods were compared (tab. 6), an acceptable agreement was demonstrated. As in experiments with total phenytoin, however, evidence was obtained that in the case of uraemia EMIT yields erroneous results. In the case investigated, the bias amounted to +109% as compared with capillary GC (tab. 5). After addition of ammonium sulphate, extraction of the ultrafiltrate of the uraemic patient by chloroform and reconstitution of the dry residue by a drug-free ultrafiltrate, the results obtained by EMIT and FPIA agreed better (2.0 and 1.6 μmol/l respectively).

Tab. 5. Determination of phenytoin in specimens from an uraemic patient treated with the drug.

	Specimen I Phenytoin (total)		Specimen II Phenytoin (total)		Specimen III Phenytoin (total)		Specimen III Phenytoin (free)	
	(μmol/l)	(%)[1])	(μmol/l)	(%)[1])	(μmol/l)	(%)[1])	(μmol/l)	(%)[1])
GC	20.8	–	24.4	–	22.2	–	3.2	–
EMIT[3])	38.4	+85	29.7	+22	32.9	+48	6.7	+109
FPIA[4])	21.0	+1	22.6	−7	21.4	−4	2.8	−12
NIIA[5])	41.0	+97	41.0	+68	47.6	+114	N[2])	N[2])
SLEFIA[6])	31.7	+52	36.5	+49	32.9	+48	N[2])	N[2])

[1]) %: Deviation in % as compared with GC.
[2]) N: Not determined; concentration below measuring range.
[3]) Lot L 03. [4]) Lot 49551. [5]) Lot L 206015. [6]) Lot 900 5032.
Specimen I: Before dialysis (creatinine: 988 μmol/l serum).
Specimen II: After dialysis.
Specimen III: Before dialysis (creatinine: 1322 μmol/l serum).

Tab. 6. Comparison of the results obtained by various immunoassays and GC for the determination of free phenytoin in samples from patients treated with the drug.

Methods (y)	vs.	(x)	n[1])	Standardized principal component analysis Slope[7])	Intercept (μmol/l)	$s_{y\cdot x}$[2]) (μmol/l)	$\bar{y}$[3]) (μmol/l)	$\bar{x}$[4]) (μmol/l)	t[5])	Correlation coefficient
EMIT	vs.	GC	39	1.02	−0.2	0.48	4.0	4.2	1.18	0.96
FPIA	vs.	GC	27	0.96	0.0	0.45	4.1	4.3	1.38	0.93
FPIA	vs.	EMIT[6])	47	0.86**	0.3	0.32	3.7	4.0	2.33*	0.98

[1]) n: Number of contributing pairs of results.
[2]) $s_{y\cdot x}$: standard error of residues.
[3]) $\bar{y}$: Mean value of method y.
[4]) $\bar{x}$: Mean value of method x.
[5]) t: t-value (paired t-test); *: significance of the bias $\bar{x}-\bar{y}$ ($p \leq 0.05$).
[6]) Original procedure performed by use of an AutoLab 5000 System.
[7]) **: significant deviation from identity (t-test: $p \leq 0.05$).

Specificity and interference

Cross-reactivity of substances with the immunochemical determination of phenytoin is shown in table 7. These compounds are structurally related to phenytoin or frequently applied concomitantly with phenytoin. Among the compounds listed only the phenytoin metabolite (5-(4-hydroxyphenyl)-5-phenylhydantoin) showed a distinct interference.

The immunoassays were susceptible to interference by extremely lipaemic sera (tab. 8). The interference is most pronounced in EMIT and NIIA. With EMIT all values differed by more than 10%, whereas the Auto ICS rejected 3 out of 5 samples. Haemolysed samples (haemoglobin ≤ 7.7 g/l) or icteric samples (bilirubin ≤ 450 μmol/l) were measured with adequate accuracy.

Tab. 7. Cross-reactivity of compounds in serum with various immunoassays for determination of phenytoin.

Compound	EMIT[1]) (μmol/l)[5])	FPIA[2]) (μmol/l)[5])	NIIA[3]) (μmol/l)[5])	SLFIA[4]) (μmol/l)[6])
Phenytoin	20	20	20	20
5-(4-Hydroxyphenyl)-5-phenylhydantoin	338	186	67	37
Carbamazepine	>4230	>4230	>4230	>4230
Clonazepam	> 320	> 320	> 320	> 320
Ethosuximide	>7080	>7080	>7080	>7080
Phenobarbital	>4310	>4310	>4310	>4310
Primidone	>4580	>4580	>4580	>4580
Valproic acid	>6930	>6930	>6930	>6930

[1]) Determinations by EMIT adapted to an Eppendorf analyzer 5010. Lot Nr. LO3.
[2]) Lot Nr. 33−499 HQ.
[3]) Lot Nr. C 105013.
[4]) Lot Nr. 900 40 12.
[5]) Concentration of a compound added to drug-free pooled human serum, which is necessary to produce a quantitation error equivalent to 20 μmol/l phenytoin.
[6]) Concentration of a compound necessary to produce a quantitation error equivalent to 20 μmol/l phenytoin in pooled human serum containing 40 μmol/l phenytoin.

Tab. 8. Interference by endogenous compounds with various immunoassays for the determination of phenytoin.

	Haemolytic[1]) (%)[8])	Icteric[2]) (%)[8])	Lipaemic I[3]) (%)[8])	Lipaemic II[4]) (%)[8])	Lipaemic III[5]) (%)[8])	Lipaemic IV[6]) (%)[8])	Lipaemic V[7]) (%)[8])
EMIT	−5	− 6	−14	−19	−24	−26	−31
FPIA	−2	− 6	− 3	+ 2	− 9	−10	−11
NIIA	−3	− 1	− 4	− 6	R[9])	R[9])	R[9])
SLFIA	−8	+21	+ 4	+ 9	+16	+26	+31

[1]) Haemoglobin: 7.7 g/l.
[2]) Bilirubin: 450 μmol/l.
[3]) Triglycerides: 7.7 mmol/l; cholesterol: 7.1 mmol/l.
[4]) Triglycerides: 13.4 mmol/l; cholesterol: 9.3 mmol/l.
[5]) Triglycerides: 18.8 mmol/l; cholesterol: 11.2 mmol/l.
[6]) Triglycerides: 23.3 mmol/l; cholesterol: 13.7 mmol/l.
[7]) Triglycerides: 30.9 mmol/l; cholesterol: 19.0 mmol/l.
[8]) Deviation in % from the target value (39.6 μmol/l).
[9]) Samples rejected by ICS.

Discussion

From the data presented it is concluded that all methods evaluated are sufficiently precise and accurate for monitoring total phenytoin concentrations in the serum of nonuraemic patients. In uraemic patients treated with phenytoin, however, only FPIA besides GC yielded reliable results for the determination of total and free phenytoin, as also shown by *Green* et al. (12). There was no evidence of interference in the immunoassays by endogenous compounds accumulated in renal failure. The same conclusion was drawn by other authors (13, 14, 15) from their experiments. Only *Toseland* et. al. (16) reported a distinct interference in the EMIT-assay by spiked uraemic sera. It seems more likely that the erroneous results are due to some metabolites of phenytoin that are accumulated in renal failure. In uraemic patients the concentration of 5-(4-hydroxyphenyl)-5-phenylhydantoin-glucuronide (HPPH-glucuronide) is 10 times higher (27–101 μmol) than in nonuraemic patients (2.7–10.1 μmol/l), whereas the small concentrations of the unconjugated metabolite (0.1–0.8 μmol/l) remain unaffected by renal function (17).

From these data and from the evaluation of the specificity it seems that 5-(4-hydroxyphenyl)-5-phenylhydantoin is not the main interfering metabolite. Some authors have presented results indicating that interference is due to 5-(4-hydroxyphenyl)-5-phenylhydantoin-glucuronide in uraemia (12, 13, 18), whereas other investigators deny that this metabolite is mainly responsible for the erroneous results obtained in some of the immunoassays (19, 20). It remains to be tested whether another metabolite of phenytoin, e.g. 5-(3,4-dihydroxy-1,5 cyclohexadien-1-yl)-5-phenylhydantoin, is responsible for the observed discrepancies.

The results demonstrate that careful selection of methodology is necessary for the determination of total and free phenytoin in the case of uraemic patients treated with the drug.

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