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Spectrometric Determination of Urokinase in Urine after Gel Filtration, Using the Chromogenic Substrate S-2444

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Summary: Using the chromogenic substrate S-2444, the spectrometric determination of urokinase (EC 3.4.21.31) in urine subjected to gel filtration was evaluated. Pure urokinase solutions were used to standardize analytical conditions. A low molecular mass (relative molecular mass < 5000) heat resistant (60 min, 97 °C) activity could be removed from urine by gel filtration (Sephadex G 25 Medium). In the analysis of the high molecular mass fraction (relative molecular mass > 5000) of urine, amidolysis remained linear during a period of 3 hours. The relation between enzyme activity and substrate turnover was linear in the range from 0.05–12 U/l. The coefficients of variation for within-run precision ranged from 1.4–3.8%. The analytical recovery was 98–104%. Average urokinase excretion in morning urines (collection period 1–3 hours) of 10 healthy males and 10 healthy females was respectively 0.82 and 0.68 U/g creatinine.

Spektrometrische Bestimmung von Urokinase mit dem chromogenen Substrat S-2444 im Harn nach Gelfiltration

Zusammenfassung: Es wurden Untersuchungen zur spektrometrischen Bestimmung der Urokinaseaktivität (EC 3.4.21.31) mit dem chromogenen Substrat S-2444 im Harn nach Gelfiltration vorgenommen. Zur Standardisierung der analytischen Bedingungen dienten reine Urokinaselösungen. Die Probenvorbereitung durch Gelfiltration (Sephadex G-25 Medium) ermöglichte die Abtrennung einer kleinmolekularen (< 5000 relative Molmasse), nicht hitzeinaktivierbaren (60 min, 97 °C) Aktivität aus dem Nativharn. Bei Verwendung der großmolekularen Fraktion aus Harn (> 5000 relative Molmasse) verlief die Amidolyse über 3 Stunden linear. Im Bereich von 0,05–12 U/l bestand eine lineare Beziehung zwischen Enzymaktivität und Substratumsatz. Für die Präzision in der Serie betrugen die Variationskoeffizienten 1,4–3,8%. Die analytische Wiederfindung lag bei 98–104%. Bei Untersuchung von je 10 gesunden männlichen und weiblichen Personen betrug die durchschnittliche Urokinaseausscheidung im Morgenharn (Sammelperiode 1–3 Stunden) 0,82 bzw. 0,68 U/g Kreatinin.

Introduction

The direct spectrometric determination of urokinase (EC 3.4.21.31), an endogenous plasminogen activator excreted in urine (1), with chromogenic substrates (2, 3) offers new diagnostic possibilities (4, 5, 6). Like other proteolytic enzymes the urokinase catalyzes the amidolytic cleavage of specially synthesized peptide-*p*-nitroanilide derivatives (7), producing coloured *p*-nitroaniline which is easily determined at 405 nm. At present the substrates Chromozym® UK (Bz-Val-Gly-Arg-*p*-nitroanilide · HCl), S-2227 (H-Glu-Gly-Arg-*p*-nitroanilide · 2 HCl) and S-2444 (< Glu-Gly-Arg-*p*-nitroanilide · HCl) are most suited for the amidolytic determination of the urokinase activity in purified preparations and in urine. Due to methodological difficulties, however, measurement in untreated urine present their own problems (4). This communication summarizes the results of our studies in order to evaluate the determination of urokinase with the chromogenic substrate S-2444 in urine subjected to gel filtration.

Materials and Methods

Reagents

Chromogenic substrate S-2444 (Kabi Diagnostica, S-11287 Stockholm): Substrate (25 mg) was dissolved in 16.7 ml of distilled water. Solution of substrate (3 mmol/l) was kept in aliquots of 1 ml in sterile polystyrene tubes, 16 × 100 mm (Greiner, D-7440 Nürtingen) in the dark at 2–8 °C. Under these conditions the solution was stable for at least one month.

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Buffer (pH 8.8 at 25 °C, μ = 0.05): 6.1 g (50 mmol/l) Tris (hydroxymethyl)-aminomethane GR (E. Merck, D-6100 Darmstadt) and 2.2 g (38 mmol/l) sodium chloride GR (E. Merck, D-6100 Darmstadt) were dissolved in 800 ml of distilled water. pH was adjusted to 8.8 at 25 °C by adding an appropriate amount hydrochloric acid (1 mol/l, Titrisol®, E. Merck, D-6100 Darmstadt). The solution was adjusted to 1000 ml with distilled water and pH was checked. 100 ml portions of buffer solution were stable for at least two months, when stored at 2–8 °C.

Solvent for urokinase standard preparation: 0,5 g polyethyleneglycol, type 6000 (Serva, Feinbiochemica, D-6900 Heidelberg) were dissolved in 100 ml distilled water within 15 min at 37 °C.

Acetic acid GR (E. Merck, D-6100 Darmstadt): 8.33 mol/l.

Aprotinin: Trasylol® (Bayer, D-5090 Leverkusen): 133 Kallikrein Inactivator Units aprotinin were added to 10 ml of buffer for use in the spectrometric assay.

Reagents and preparation methods were in accordance with recommendations of the substrate manufacturer.

Urokinase solution

Urokinase assay standard (Serono, D-7800 Freiburg i. Br.): Lyophilized content of one vial (stated activity 2609 ± 30.04 CTA U[1]) was reconstituted in 10 ml of solvent (5 °C).

Stock solution was either used immediately or aliquots of 0.5 ml were filled into sample cups (Sarstedt, D-5223 Nümbrecht, Ref. No. 73641) within 5 min and frozen at −28 °C. Frozen stock solution was thawed within 5 min at 37 °C. Freezing, thawing and storage at −28 °C over a period of at least one month did not alter activity. Appropriate dilutions of stock solution were also prepared by addition of solvent (5 °C). If no gel filtration was performed, samples were diluted with buffer (10 °C) immediately prior to analysis, so that the conditions resembled those after gel filtration.

Specimen

Collection and handling: Urokinase in urine is stable in plastic containers for six hours at room temperature, even without correction of the pH (4). After an initial voiding, urine was collected in a plastic bottle over a 3 hour morning period from 06.00–09.00 a.m. Immediately after collection, urine was centrifuged in polystyrene tubes, 16 × 100 mm (Greiner, D-7440 Nürtingen) for 10 min at 1500 *g*. When untreated urine was analyzed, the sample was diluted with buffer (10 °C) corresponding to the conditions after gel filtration.

Equipment

Digital Micro Balance 2406 (Sartorius, D-3400 Göttingen).

Labofuge® III (Heraeus-Christ, D-3360 Osterode).

Water Bath, type 3041 (Köttermann, D-3165 Hänigsen).

pH Meter 22 (Radiometer, DK-2830 Kopenhagen).

Digital photometer 6114 S (Eppendorf Gerätebau, Netheler + Hinz GmbH, D-2000 Hamburg).

Methods

Gel filtration

Column PD-10, Sephadex G-25 Medium (Pharmacia Fine Chemicals AB, S-75104 Uppsala) was equilibrated with 50 ml of buffer. 2.5 ml of sample were added and eluted with 3.5 ml (fraction relative molecular mass > 5000) or 9.5 ml of buffer (fraction relative molecular mass > 5000 plus < 5000) respectively. Gel filtration was performed at room temperature. To prevent loss of activity, the eluate was collected in a polystyrene tube, 16 × 65 mm (Greiner, D-7440 Nürtingen) kept in ice. All eluates were analyzed within 6 min after completion of gel filtration.

Spectrometric determination

The assay was performed in a reaction tube (Brand, D-6980 Wertheim, Cat. No. 780500) at 37 °C. 0.3 ml of sample (10 °C) was added to 0.6 ml of buffer (37 °C) containing aprotinin. After a 3 min preincubation period the reaction was started by addition of 0.1 ml of substrate solution (37 °C). Reactions were stopped by adding 0.1 ml of acetic acid after 15 and 180 min. Methodological modifications are indicated separately. For the determination of the sample blank, acetic acid and substrate solution were added after completion of the reaction time. In order to detect spontaneous hydrolysis of substrate, additional reagent blanks were run. Readings of absorbance were performed at 405 nm (light path 10 mm) against water. Generated colour remained stable for at least 4 hours.

Urokinase activity in U/l was calculated using a value of 1040 m^2 mol^{-1} for the molar lineic absorbance of *p*-nitroaniline. All activity measurements were performed in duplicate.

Heat resistant activity

For assessment of heat resistant activity the eluates or the appropriately diluted samples were heated in polypropylene tubes, 16 × 100 mm (Greiner, D-7440 Nürtingen) for 60 min at 97 °C.

Precision

Within-run precision was calculated from the differences between duplicates (8). Each of the analyzed urine specimens was subjected to a fivefold gel filtration.

Accuracy

Accuracy was assessed by adding 0.5 ml of urokinase solution (1.55 U/l, 10 °C) or 0.5 ml of buffer (10 °C) to the investigated eluates.

Urokinase excretion

For calculation of urokinase excretion in U/g creatinine, the determination of creatinine concentration (9) in urine was performed with the AutoAnalyzer® I (Technicon Instruments Corporation, Tarrytown, N.Y.).

Results

Analytical characteristics

Employing urokinase solutions (1000–50000 CTA U/l), a linear relation was found between the enzyme activity and the substrate turnover (fig. 1). When the assay was performed with a constant enzyme activity (10000 CTA U/l), the amidolysis remained linear during reaction times from 5 to 180 min (fig. 2).

Recovery after gel filtration

An average recovery of 94.3% was observed after gel filtration of 10 urokinase solutions (fig. 3). The mean values ($\bar{x}$) of urokinase activity were 1.92 U/l ($s_{\bar{x}}$ = 0.37 U/l) before and 1.81 U/l ($s_{\bar{x}}$ = 0.35 U/l) after gel filtration (fraction relative molecular mass > 5000). When

[1]) A CTA unit refers to the standard urokinase unit adopted by the Committee on Thrombolytic Agents, Nat. Inst. of Health, Bethesda, Md. USA.

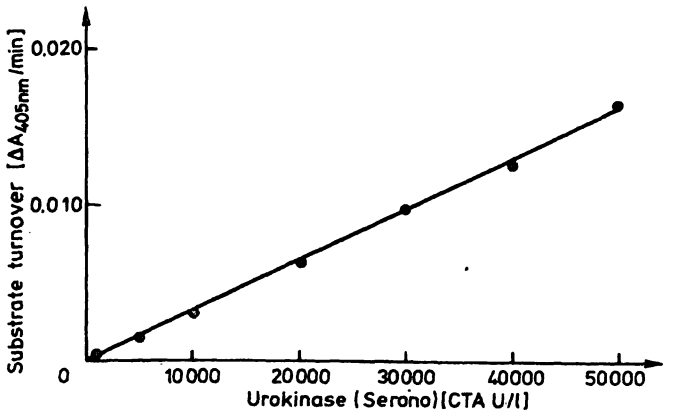

Fig. 1. Relation between enzyme activity (urokinase assay standard solution) and substrate turnover (chromogenic substrate S-2444, reaction time 15 min).
• $\bar{x}$ of n = 2

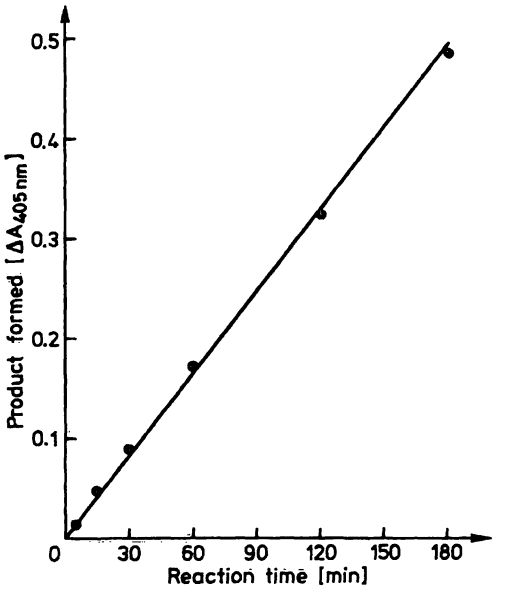

Fig. 2. Relation between reaction time and substrate turnover (chromogenic substrate S-2444, urokinase assay standard solution, 10000 CTA U/l).
• $\bar{x}$ of n = 3

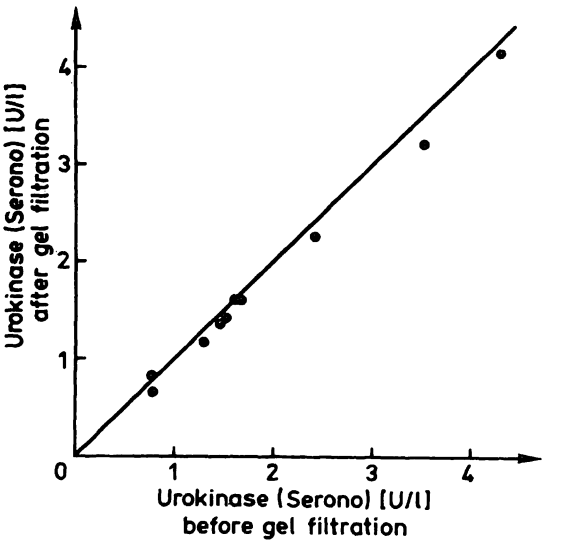

Fig. 3. Recovery of enzyme activity after gel filtration (Sephadex G-25 Medium) of urokinase assay standard solutions (n = 10, chromogenic substrate S-2444, reaction time 15 min).
—— regression line y = x

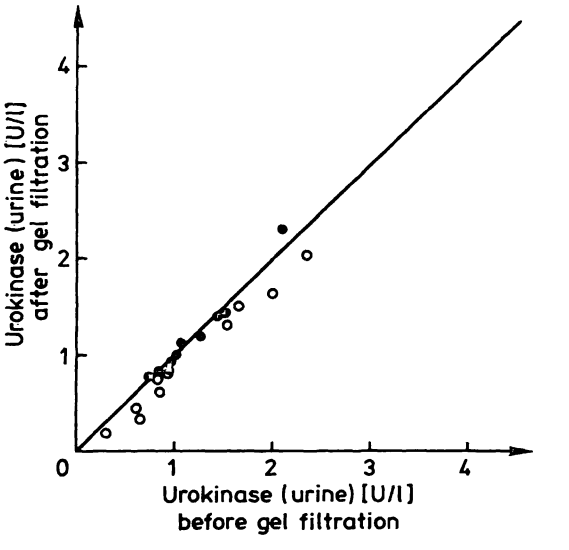

Fig. 4. Recovery of urokinase activity after gel filtration (Sephadex G-25 Medium) of 20 urines (chromogenic substrate S-2444, reaction time 180 min).
o fraction of relative molecular mass > 5000 (n = 10 urines)
• fraction of relative molecular mass > 5000 plus < 5000 (n = 10 urines)
—— regression line y = x

10 urines were analyzed, systematically lower enzyme activities were found in the high molecular fractions (relative molecular mass > 5000) after gel filtration (fig. 4). The average recovery amounted to 84.4% ($\bar{x}$ = 1.15 U/l, $s_{\bar{x}}$ = 0.21 U/l before and $\bar{x}$ = 0.97 U/l, $s_{\bar{x}}$ = 0.20 U/l after gel filtration). From the combined high and low molecular fractions (relative molecular mass > 5000 plus < 5000) of a second group of 10 urines, an average recovery (fig. 4) of 98.3% was observed ($\bar{x}$ = 1.20 U/l, $s_{\bar{x}}$ = 0.13 U/l before and $\bar{x}$ = 1.18 U/l, $s_{\bar{x}}$ = 0.14 U/l after gel filtration). To further characterize the activity present in the low molecular fraction of urine, the urokinase solutions and both urine fractions were tested before and after heating for 60 min at 97 °C. 98–100% of the activity present in the urokinase solutions was inactivated by heating (tab. 1). In 5 urines (tab. 2), 94.8–98.4% of the heat labile activity was demonstrable in the high molecular, and 1.6–5.2% in the low molecular fraction. The proportion of the heat resistant activity in the high molecular fraction amounted to only 0.01–0.06 U/l.

Tab. 1. Activity of urokinase solutions before and after heating (60 min, 97 °C).

Urokinase solution (Urokinase assay standard, Serono) No.	Urokinase activity (EC 3.4.21.31) before heating [U/l]	after heating [U/l]	Heat-labile activity [%]
1	1.32	0.000	100
2	1.64	0.016	99
3	2.63	0.000	100
4	3.16	0.032	99
5	3.98	0.000	100
6	4.20	0.000	100
7	4.61	0.092	98

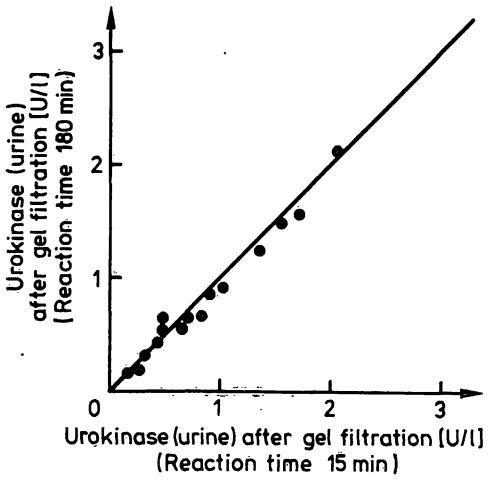

Fig. 5. Recovery of urokinase activity (chromogenic substrate S-2444) in 15 urines subjected to gel filtration (Sephadex G-25 Medium) after prolongation of reaction time from 15 to 180 min.
—— regression line y = x

Reliability of urokinase determinations in urine subjected to gel filtration

Compared with the activity measured at 15 min, the activity measured after 180 min incubation (fig. 5) was 6% lower for simultaneous determinations on the high molecular fractions of 15 urines (after 15 min $\bar{x}$ = 0.84 U/l, $s_{\bar{x}}$ = 0.16 U/l, after 180 min $\bar{x}$ = 0.79 U/l, $s_{\bar{x}}$ = 0.15 U/l). A linear relation could be demonstrated between the sample volumes and the substrate turnover at reaction times of 15 and 180 min, when 0.1–0.3 ml of sample were employed in the assay (fig. 6). The coefficients of variation for the within-run precision ranged from 1.4–3.8% (tab. 3). The recovery was 98.2–104% after addition of 1.55 U/l urokinase (tab. 4).

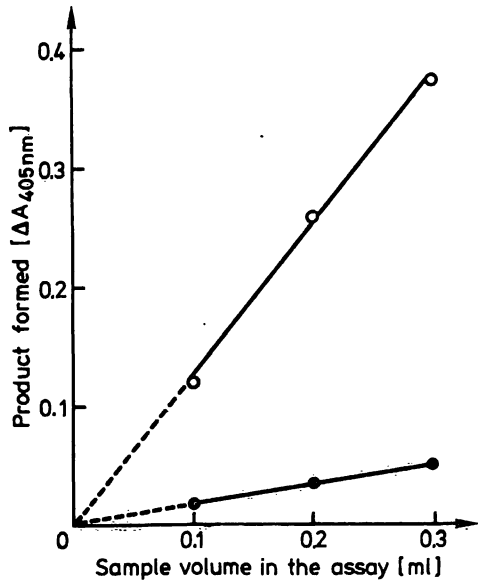

Fig. 6. Relation between sample volume (urine subjected to gel filtration, Sephadex G-25 Medium) and substrate turnover (chromogenic substrate S-2444).
• $\bar{x}$ of 10 urines (reaction time 15 min)
○ $\bar{x}$ of 10 urines (reaction time 180 min)

Preliminary reference values for urokinase excretion in urine

When morning urines (collection period 1–3 hours) of 20 healthy individuals (10 males, median of age 37 years, range 32–46 years; 10 females, median of age 34 years, range 26–53 years) were analyzed, the observed urokinase activities ranged from 0.18–2.57 U/l. The average urokinase excretion in U/g creatinine in urine amounted to 0.82 U/g (range 0.43–1.07) in the males and 0.68 U/g (range 0.42–1.10 U/g) in the females.

Tab. 2. Amidolytic activity before and after heating (60 min, 97 °C) of high and low molecular fractions of urines subjected to gel filtration (Sephadex G-25 Medium).

Urine	Amidolytic activity Fraction relative molecular mass				Heat-labile activity Fraction relative molecular mass		
	> 5000 before heating	> 5000 after heating	< 5000 before heating	< 5000 after heating	> 5000 + < 5000	> 5000	< 5000
No.	[U/l]	[U/l]	[U/l]	[U/l]	[U/l]	[%]	[%]
1	1.31	0.03	0.20	0.13	1.35	94.8	5.2
2	1.20	0.06	0.18	0.13	1.19	95.8	4.2
3	1.61	0.04	0.16	0.13	1.60	98.1	1.9
4	1.87	0.03	0.13	0.10	1.87	98.4	1.6
5	0.61	0.01	0.08	0.06	0.62	96.8	3.2

Tab. 3. Within-run precision of spectrometric determination of urokinase with chromogenic substrate S-2444 in urine subjected to gel filtration (Sephadex G-25 Medium).

Urine subjected to gel filtration No.	Number of determinations (Duplicates) n	Urokinase activity (EC 3.4.21.31) $\bar{x}$ [U/l]	Coefficient of variation [%]
1	5	0.48	3.8
2	5	0.72	3.6
3	5	0.87	2.1
4	5	1.41	2.2
5	5	2.34	1.4
6	5	2.66	1.4

Tab. 4. Accuracy of spectrometric determination of urokinase with chromogenic substrate S-2444 in urine subjected to gel filtration (Sephadex G-25 Medium).

Urine subjected to gel filtration No.	Urokinase activity (EC 3.4.21.31) after addition of 1.55 U/l expected [U/l]	measured [U/l]	Deviation from expected value [Δ%]
1	2.17	2.14	−1.4
2	2.21	2.17	−1.8
3	2.41	2.47	+2.5
4	2.50	2.60	+4.0
5	3.79	3.88	+2.4

Discussion

At the present time the chromogenic substrate S-2444 is the substrate of choice for the determination of urokinase (10, 11). Using urokinase solutions the linearity was assessed (fig. 1 and 2) in the low range of activity from 1000–50000 CTA U/l, corresponding to 0.16–8.0 U/l, because activities of 0.50–0.90 U/l were observed in the morning urines of healthy individuals with the substrate Chromozym® UK (5). To increase the sensitivity, 0.3 ml of diluted sample were employed in the assay. This was in contrast to the substrate manufacturer's original laboratory instructions, which recommend a sample volume of 0.1 ml. The dilution of the sample (factor 1.4) corresponded with the conditions after gel filtration. In addition the reaction time was prolonged from the original 5 min to 15 and 180 min respectively. The molar absorbance of *p*-nitroaniline depends on the reaction conditions (12). At present the applied value of 1040 $m^2 \cdot mol^{-1}$ for the molar lineic absorbance of *p*-nitroaniline seemed to be a suitable compromise.

The average recovery in the macromolecular fraction after gel filtration of 94.3% for the original activity of pure urokinase solutions (fig. 3) is acceptable. For the column PD-10, Sephadex G-25 Medium, a recovery of 95.3% is stated by the manufacturer, if human serum albumin (25 mg) dissolved in NaCl solution (0.5 mol/l, 2.5 ml) is used. The distinctly lower average recovery of enzyme activity (84.4% of the activity in the untreated urines) observed in the high molecular urine fractions (fig. 4) was unexpected. The reported values (1) for the relative molecular masses of urokinase excreted in urine are 31500 (S_1-type) and 54700 (S_2-type). Therefore it was supposed, that a low molecular activity (relative molecular mass < 5000) must be present in the untreated urine. This was confirmed by the average recovery of 98.3% of the original activity, when the combined high and low molecular fractions of urines were investigated (fig. 4). In contrast to the activities present in the urokinase solutions and in the high molecular fractions of urines (tab. 1 and 2), the activity of the low molecular fraction of urine (tab. 2) could not be inactivated by heating for 1 hour at 97 °C, as reported recently (13). The nature of the low molecular, heat resistant activity is unknown. A non enzymatic interference, e.g. from salts or urochromes, is possible.

For the determination of urokinase in urine subjected to gel filtration, analytical conditions identical to those chosen for the urokinase solution were applied. The activities measured simultaneously after reaction times 15 and 180 min differed to an extent, which is negligible for practical purposes (fig. 5). The observed linearity of the amidolysis for the high molecular urine fraction within 3 hours corresponds with the results of other investigators for the analysis of untreated urine using the substrate Chromozym® UK (4, 6). For reaction times of 15 and 180 min, the increase of the sample volume from 0.1 ml–0.3 ml caused no loss of linearity in the range 0.54–1.62 U/l, or 0.34–1.02 U/l (fig. 6). The results presented indicate a linear relation between the substrate turnover and the enzyme activity in the range 0.05–12.2 U/l, if 0.3 ml of sample and a reaction time of 180 min are employed. Aprotinin was added to increase the specifity (4, 5). The within-run precision and the accuracy of the urokinase measurements in urine subjected to gel filtration were comparable to those of serum enzyme determinations.
The reference values for the urokinase excretion in urine reported here are considered to be preliminary. Conclusions regarding sex related differences of urokinase output should not be drawn from these data. The urokinase excretion was calculated in U/g creatinine, in order to compensate for the influence of different volumes of urine.

The spectrometric determination of urokinase with the chromogenic substrate S-2444 represents a practicable method for the measurement of urokinase activity in pure solutions and in urine subjected to gel filtration. By gel filtration, a low molecular heat resistant activity can be removed from the untreated urine. As with other determinations of enzyme activities in urine (14, 15), the additional work load of gel filtration is therefore considered to be acceptable. Comparative investigations

with the conventional methods for the determination of the urokinase activity are still lacking. A final judgement on the diagnostic significance of the amidolytic measurement of the urokinase activity in the field of urine enzymology (16) can not be passed until greater populations of patients have been investigated.

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