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ANALYSIS OF URINARY 3-METHOXY-4-HYDROXYPHENYLGLYCOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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SUMMARY

A high-performance liquid chromatographic method with electrochemical detection for the quantitation of total 3-methoxy-4-hydroxyphenylglycol (MHPG) in human urine is described. Existing methods for deconjugation and extraction have been optimized. The present method is simpler than existing methods with a high precision. Urinary MHPG is deconjugated enzymatically and subsequently extracted with ethyl acetate. The organic layer is extracted with acetic acid and a sample of the aqueous layer is injected into a reversed-phase column. In one run 90 samples can be processed. The critical parameters of deconjugation, extraction and chromatography are described. Data for reproducibility and selectivity are presented.

INTRODUCTION

The catecholamines play an important role in the function of both the central and the peripheral nervous systems. Therefore, extensive attention has been paid to the analysis of catecholamines and their metabolites in tissue extracts and body fluids. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been shown to be particularly suitable for these compounds [1]. Simple methods for the quantification of adrenaline, noradrenaline, dopamine, and the metabolites vanillylmandelic acid, homovanillic acid, metanephrine and normetanephrine in tissue extracts, cerebrospinal fluid and urine have been presented [1—4].

However, the analysis of the noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) with HPLC and ED apparently meets with more difficulties, especially so when applied to the measurement of urinary MHPG [5]. A simpler method for analysis of urinary MHPG has been described for the

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diagnosis of neural-crest tumors [6,7] but the precision of this procedure has not been described.

We were interested in the urinary excretion of MHPG in psychiatric patients, where relatively small differences are observed between several diagnostic groups of patients [8]. This demands a method with high precision, which is simple enough to be reliable in routine use. We describe here such a method and characterize the sample preparation procedure, as well as the precision and selectivity of the method.

EXPERIMENTAL

Apparatus

The analyses were performed on a Hewlett-Packard 1084B chromatograph with an electrochemical detector (Bioanalytical Systems), consisting of an LC-4 controller and a TL-5 cell (glassy carbon electrode and Ag/AgCl reference electrode). Prepacked reversed-phase columns were used. An analytical column, 15 cm × 4.6 mm I.D., packed with Hypersil ODS, 5-μm particle size, was obtained from Chrompack (Middelburg, The Netherlands), and combined with a 3 cm × 4.6 mm I.D. precolumn, packed with LiChrosorb C_{18}, 10-μm particle size (Brownlee Labs., Santa Clara, CA, U.S.A.), when urine samples were analysed.

Peak areas were obtained using the integrator of the Hewlett-Packard chromatograph. The reliability of peak areas in quantifying MHPG appeared to be equal to or better than the reliability of peak heights.

Chromatographic conditions

The flow-rate was adjusted to 2 ml/min and the temperature of the column compartment and of the eluents was 30°C. Eluent A contained 0.05 mol/l Na_2HPO_4 and 1.34 mmol/l disodium EDTA, dissolved in distilled water and acidified with perchloric acid to pH 3. Eluent B was identical except for the use of 5% (v/v) propanol as solvent. The eluents were filtered through Millipore membrane filters of pore size 0.45 μm.

The electrochemical detector was operated at +0.8 V vs. Ag/AgCl.

Materials

For standards and internal standards bis-(4-hydroxy-3-methoxyphenylglycol) piperazine (MHPG; Sigma, St. Louis, MO, U.S.A.), 4-hydroxy-3-methoxyphenylglycol sulphate ester (MHPG-SO_4; Fluka, Buchs, Switzerland), 3,4-dihydroxyphenylglycol (DHPG, Labkemi, Stockholm, Sweden), 3,4-dihydroxyphenylglycol ethanol (DOPET, Labkemi), and 4-hydroxy-3-methoxyphenyl ethanol (MOPET, Labkemi) were used. Glusulase (β-glucuronidase—arylsulphatase) from *Helix pomatia* was purchased from Boehringer (Mannheim, G.F.R.). All other chemicals were reagent grade. Water was demineralized and distilled in an all-glass apparatus.

Stock solutions (ca. 100 ppm) of standards were prepared in 0.05 *M* $HClO_4$ with 0.05% (w/v) disodium EDTA and 0.05% (w/v) $Na_2S_2O_5$ and stored at 4°C for two weeks or less.

Urine was collected from seven patients in 12-h portions (8—20 h and

20—8 h) over 0.5 g of disodium EDTA and 0.5 g of $Na_2S_2O_5$, and from one helathy subject (reference urine). Urine was stored at —70°C for several months without loss of MHPG.

Analysis of urine samples

In each series four concentrations of both MHPG and MHPG-SO_4 standards (ca. 5—40 nmol/ml) in duplicate, urine samples in duplicate, and a sample from each of these urines with ca. 10 nmol/ml MHPG or MHPG-SO_4 added to it were analysed. The reference urine was run in all series.

To a sample of 2 ml, 200 μl of saturated $BaCl_2$ were added and the pH adjusted to 11.5—12 with 5 *M* and 1 *M* NaOH. After centrifugation (5 min, 3000 *g*) the supernatant was decanted and the pH adjusted to 6.0 with 5 *M* and 1 *M* acetic acid. Chloroform (50 μl) and 70 μl of Glusulase were added and the sample was incubated for 16—18 h at 37°C in a sealed tube. To this, excess NaCl (1—1.5 g), 0.2 g of Florisil® and 8 ml of ethyl acetate were added. The tube was shaken for 10 min and centrifuged for 5 min at 3000 *g*. From the organic layer 4 ml were taken and added to 4 ml of hexane and 2 ml of 0.1 *M* acetic acid, containing 0.05% (w/v) disodium EDTA and 0.05% (w/v) $Na_2S_2O_5$. The tube was shaken for 10 min and centrifuged for 5 min at 3000 *g*. The organic layer was aspirated off and 50 μl of the aqueous layer were used for chromatography. Elution was isocratic at 0.25% propanol for 6 min and then the propanol concentration was increased to 4.75% to remove slow components.

Peak identification was primarily by comparison of retention time with reference solutions and by addition of standard MHPG to urine samples. None of a large number of compounds structurally related to MHPG showed a similar retention time.

RESULTS AND DISCUSSION

Deconjugation

It has been reported that the extent of hydrolysis of MHPG with Glusulase varies with the urine sample, apparently due to endogenous inhibitors of the enzymes employed [9]. We therefore used a modification described by Weil-Malherbe [10] involving precipitation of anionic compounds with $BaCl_2$. The extent of hydrolysis was checked by comparing the recoveries of free MHPG and MHPG-SO_4. The recovery of MHPG standards in six experiments was 61 ± 7% (mean ± S.D.), while the recovery of MHPG-SO_4 standards in five of these experiments was also 61 ± 7% (see Table II). When standards were added to urine, the recovery of MHPG-SO_4 was 58 ± 5% (mean ± S.D., n=7) while the recovery of MHPG added to samples of the same urines was 56 ± 3%. Furthermore, there were no significant differences between the recovery of MHPG-SO_4 added to urine samples of different patients or added to different urine samples of the same patient. Although no MHPG glucuronidate was available to check its deconjugation, we conclude that $BaCl_2$ treatment of the urine samples results in complete hydrolysis of MHPG and removes variations between different urine samples.

Extraction

Sample clean-up with high selectivity for MHPG is difficult to achieve, since no functional groups are contained in the MHPG molecule upon which to focus for such procedures. The polarity of MHPG, therefore, is the only useful property. Ethyl acetate appears to be the most suitable compound for organic extraction. Mostly, multiple extractions with ethylacetate are utilized to obtain a satisfactory recovery. The large volume of organic layer is then taken to dryness and the residue reconstituted in an aqueous phase [6,11]. To increase the selectivity of the procedure, the ethyl acetate layer was extracted back with 0.1 *M* acetate, which eliminates interfering, probably acidic, compounds.

To obtain satisfactory recoveries, the conditions for this extraction procedure were optimized. Extraction of MHPG can be facilitated by manipulating the polarity of the solvents for extraction. Fig. 1 shows the effect of addition of hexane on the partition of MHPG and related compounds between ethyl acetate and acetic acid. Addition of hexane has a dual effect, namely, facilitation of extraction into acetate by decreasing the polarity of the organic solvent, and a decrease in recovery due to the increase in volume of the organic phase. Therefore, in Fig. 1 the partition coefficient k' is shown, which is corrected for loss of recovery due to the increase in volume of the organic phase (see legend). For practical reasons we chose to use equal volumes of hexane and ethyl acetate in subsequent experiments. Table I shows that

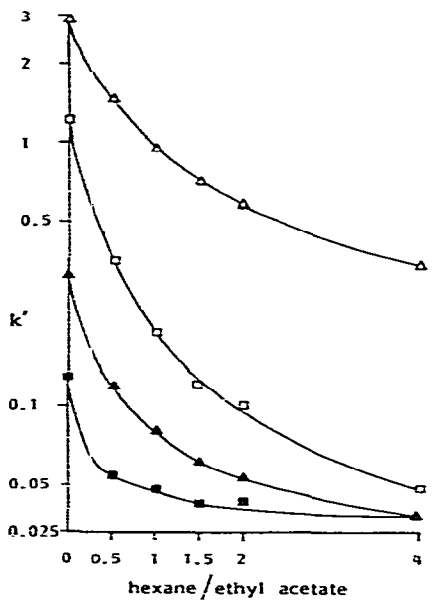


Fig. 1. Effect of hexane on the partition of MHPG and related compounds in an ethyl acetate—acetate system. Various volumes of hexane were added to 4 ml of ethyl acetate and 2 ml of 0.1 *M* acetic acid. The partition coefficient k' was calculated from $k' = (P/Q)(V_Q/V_P)$, where V_Q = 2 ml and V_P = 4 ml, also when hexane was added. Therefore, k' is the real partition coefficient k corrected for reduced extraction into acetic acid due to the increase in volume of the organic phase by addition of hexane. (△), 3-Methoxy-4-hydroxyphenylethyl alcohol (MOPET); (□), 3,4-dihydroxyphenylethyl alcohol (DOPET); (▲), 3-methoxy-4-hydroxyphenyl glycol (MHPG); (■), 3,4-dihydroxyphenyl glycol (DHPG).

TABLE I

ACETATE/ETHYL ACETATE PARTITION COEFFICIENTS OF MHPG AND RELATED METABOLITES

To calculate k_1, the partition of compounds between various volumes of ethyl acetate (2, 4, 8 and 16 ml) and 2 ml of 0.05 *M* acetate buffer (pH = 6) was measured with and without the addition of 1.5 g of NaCl to the mixture. k_2 was obtained in a similar way except that the ethyl acetate was saturated with water before use and the aqueous phase consisted of 0.1 *M* acetic acid (pH < 2). Results are expressed as mean ± S.D., n=4 (4 volumes of organic phase).

	k_1		k_2	
	—NaCl	+ NaCl	—Hexane	+Hexane (1:1, v/v)
DHPG	0.17 ± 0.04	0.34 ± 0.06	0.13 ± 0.07	0.045 ± 0.012*
MHPG	0.32 ± 0.005	1.29 ± 0.06	0.32 ± 0.013	0.077 ± 0.009
DOPET	1.56 ± 0.08	5.8 ± 0.33	1.24 ± 0.045	0.19 ± 0.007
MOPET	2.9 ± 0.14	25 ± 0.35	2.9 ± 0.14	0.95 ± 0.019

*These k_2 values are corrected for dilution by hexane (see legend to Fig. 1).

extraction into ethyl acetate is facilitated by saturating amounts of NaCl.

In addition to manipulation of the partition coefficients, the recovery of MHPG can be optimized by the choice of solvent volumes used in the extraction procedure. These volumes can be calculated from the following description of the extraction procedure.

extraction		back-extraction
P_1 (V_P)	——— transfer ———	P_2 (V_P)
↑ ↓ k_1		↑ ↓ k_2
Q_1 (V_{Q_1})		Q_2 (V_{Q_2})

where k_1 and k_2 are partition coefficients, P_1 and P_2 are amounts of compound in organic layers, Q_1 and Q_2 are amounts of compound in aqueous layers, V_P is volume of organic layer, and V_{Q_1} and V_{Q_2} are volumes of aqueous layers. Q_1 and P_1 are the amounts of compound in the aqueous and organic layers, respectively, after extraction with ethyl acetate. Assuming the original amount to be 1, then

$$P_1 + Q_1 = 1 \quad \text{or} \quad P_1 = 1 - Q_1 \tag{1}$$

and

$$k_1 = \frac{P_1}{Q_1} \cdot \frac{V_{Q_1}}{V_P} \quad \text{or} \quad Q_1 = \frac{P_1 \; V_{Q_1}}{k_1 \; V_P} \tag{2}$$

The organic phase with the amount P_1 is transferred and extracted with 0.1 *M* acetic acid, which results in the amounts P_2 and Q_2 in the organic and aqueous layers, respectively. Therefore, the recovery is Q_2:

$$Q_2 = P_1 - P_2 \tag{3}$$

and

$$k_2 = \frac{P_2}{Q_2} \cdot \frac{V_{Q_2}}{V_P} \quad \text{or} \quad P_2 = \frac{k_2\ Q_2\ V_P}{V_{Q_2}} \tag{4}$$

Combining eqns. 1 and 2 gives

$$P_1 = \frac{k_1\ (V_P/V_{Q_1})}{1 + k_1\ (V_P/V_{Q_1})} \tag{5}$$

From eqns. 3, 4 and 5 the recovery Q_2 is calculated:

$$Q_2 = P_1 - P_2 = \frac{k_1 (V_P/V_{Q_1})}{1 + k_1 (V_P/V_{Q_1})} - \frac{k_2 Q_2 V_P}{V_{Q_2}}$$

or

$$Q_2 = \frac{k_1\ V_P}{(V_{Q_1} + k_1 V_P)\ [1 + k_2 (V_P/V_{Q_2})]} \tag{6}$$

The partition coefficients k_1 and k_2 have been optimized as discussed before and shown in Fig. 1 and Table I. If we use the corrected k_2' in eqn. 6, we can neglect the volume of hexane added for back-extraction.

It appears from eqn. 6 that the recovery Q_2 can approach 1 if V_{Q_1} is decreased and V_{Q_2} is increased. Since V_{Q_2}/V_{Q_1} is also the dilution factor, the sensitivity of the method will decrease with increasing recovery. Therefore, the ratio of volumes V_{Q_1} to V_{Q_2} is chosen as 1 : 2 to obtain a high recovery with an overall sensitivity which is sufficient for the analysis of urinary MHPG.

This leaves the volume V_P of the organic phase to be optimized. Fig. 2 illustrates the effect of various volumes V_P on the recovery Q_2 for MHPG and related compounds. The optimal volume V_P can also be calculated by differentiation of eqn. 6 when $\frac{d\,Q_2}{d\,V_P} = 0$. This results in

$$V_P = \sqrt{\frac{V_{Q_1}\ V_{Q_2}}{k_1\ \ k_2}} \tag{7}$$

For the example in Fig. 2 this results in V_P = 9 ml and, using eqn. 6, in the maximal recovery Q_2 = 72.7%.

These results led to the extraction procedure for MHPG from urine as described under Experimental. This procedure is similar to the conditions illustrated in Fig. 2, except that only 4 ml of the ethyl acetate layer were used for back-extraction into 2 ml instead of 4 ml of 0.1 *M* acetic acid.

The present extraction procedure for urine is very suitable for routine use, since (1) only two extraction steps are necessary, (2) recovery is relatively high, and (3) errors in volume do not affect the recovery to a great extent. Concerning point 3, we calculated that starting with 2.5 ml of urine sample instead of 2.0 ml reduces the recovery only from 72.6% to 71.4%, when using 8 ml of ethyl acetate. This is important, since urine samples have to be adjusted to pH 11.5 and thereafter to pH 6.0 before extraction, which results

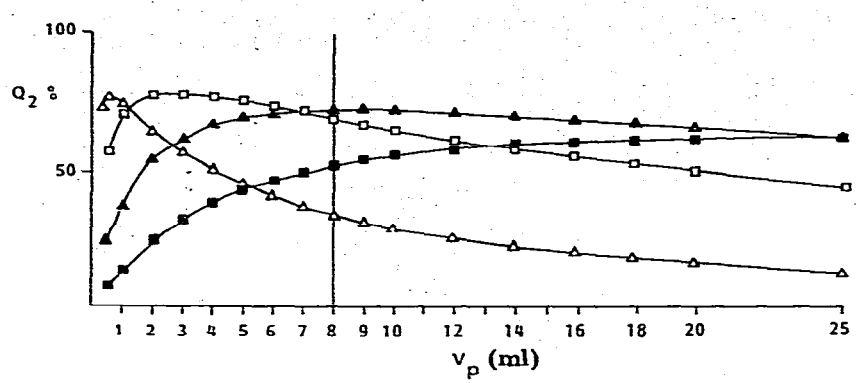


Fig. 2. Calculated recovery Q_2 of 3-methoxy-4-hydroxyphenylglycol (MHPG) and related compounds as a function of the volume V_P of the organic phase (see text).

$$Q_2 = \frac{k_1 \; V_P}{(V_{Q_1} + k_1 V_P)\,(1 + k_2 \frac{V_P}{V_{Q_2}})}$$

Table I gives k_1 and k_2 assuming the addition of NaCl and hexane (1:1). V_{Q_1} = 2 ml and V_{Q_2} = 4 ml. V_P = 8 ml is used for extraction of urine. For symbols see Fig. 1.

in the addition of different volumes of base and acid (between 0.05 and 0.2 ml in our hands).

Finally, 0.2 g of Florisil® was added to urine before extraction with ethyl acetate, since this largely prevented the formation of an interface, which disturbs the extraction [11]. Fig. 3 illustrates that addition of Florisil also resulted in a cleaner extract, while no loss of MHPG was observed.

Chromatography

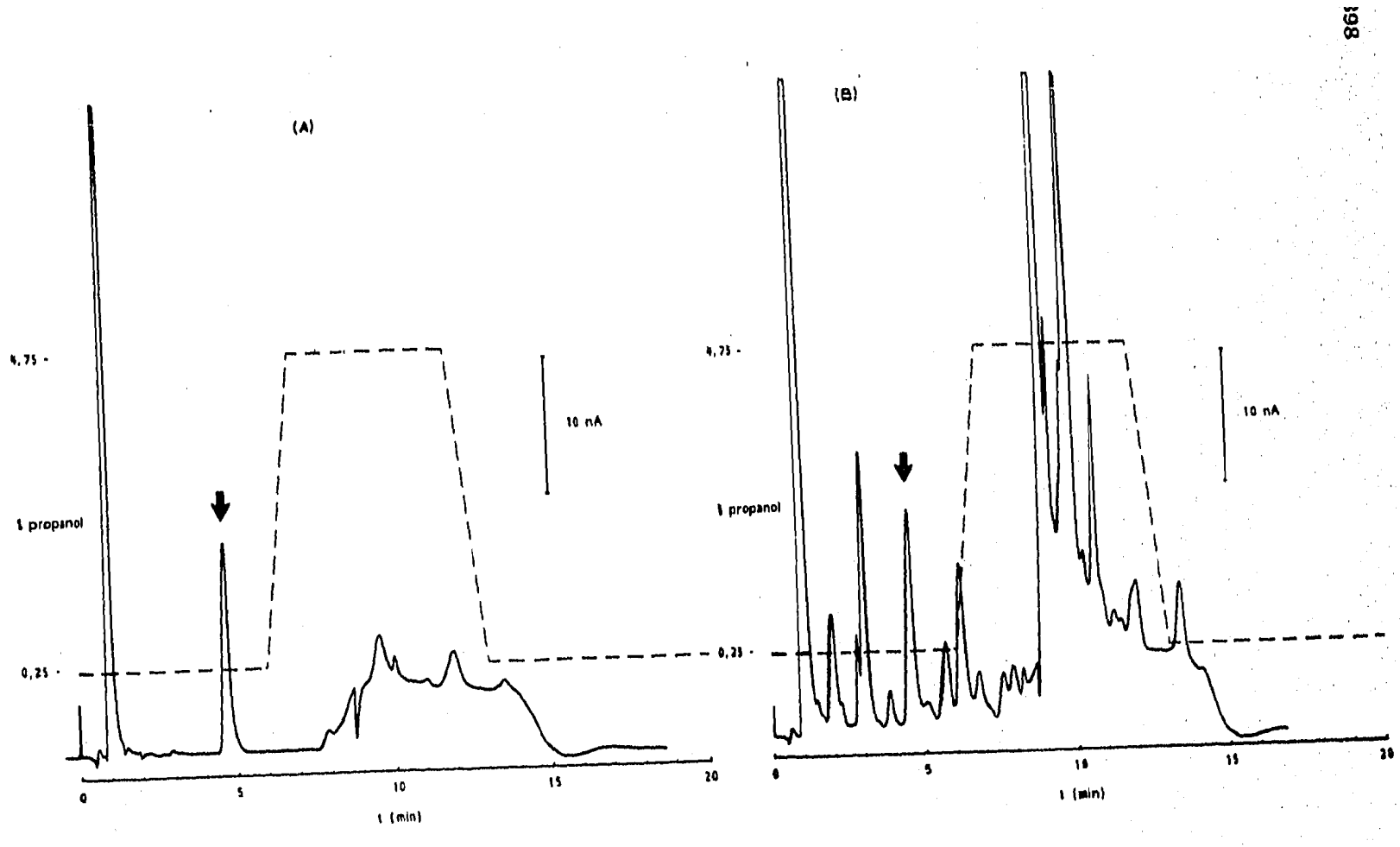
Fig. 3 shows representative chromatograms of an urine extract and a standard solution.

Fig. 4 shows that retention of MHPG cannot be modified by changing the pH of the eluents. Increasing the concentration of propanol in the eluent results in a decrease in retention time of MHPG. However, without propanol the k' for MHPG is only about 5 (Fig. 5). Therefore, separation of MHPG is best achieved with low concentrations of propanol. To speed up elution of the large number of slow components from urine, a steep gradient of propanol after elution of MHPG is useful, as is apparent from Fig. 3. It takes only about 5 min to equilibrate the column again with eluent with 0.25% propanol. The electrochemical detector used is suitable for routine use. The cell was cleaned with methanol only after several hundred analyses and polished only every few months. Over three months the absolute response of the detector to MHPG did not change significantly.

Quantitation of urinary MHPG

Calibration curves for reference MHPG and standard solutions of MHPG and MHPG-SO_4 taken through the entire procedure were linear ($r > 0.99$, n=8). Typical calibration curves are shown in Fig. 6.

We were not able to find a suitable internal standard for the analysis of


(A)
0,75
% propanol
0,25
10 nA
0
5
10
15
20
t (min)
(B)
0,75
% propanol
0,25
10 nA
0
5
10
15
20
t (min)

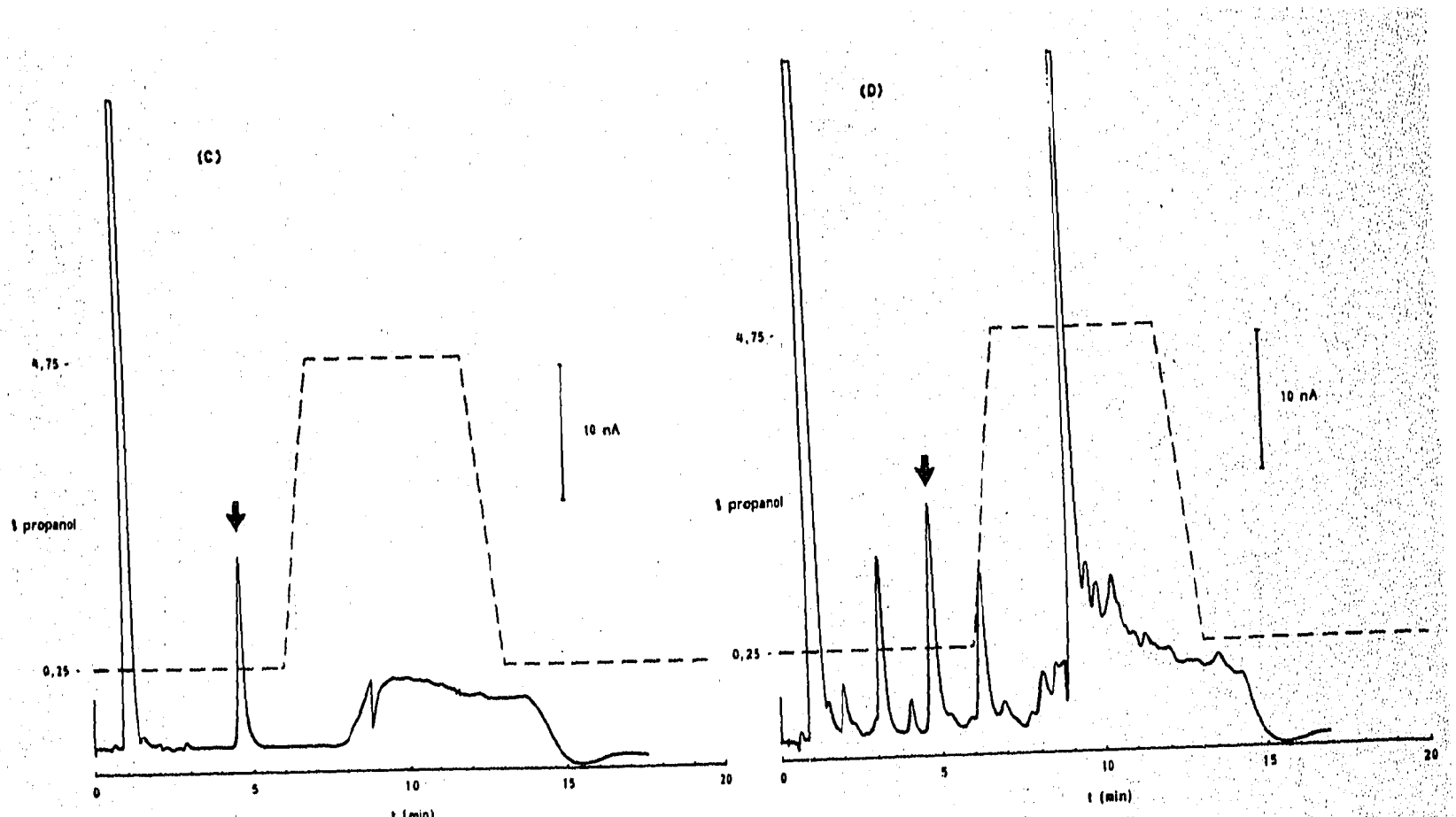


Fig. 3. Chromatography of MHPG with a discontinuous propanol gradient using ED. The mobile phase is 0.05 *M* Na_2HPO_4 containing 1.34 mmol disodium EDTA and acidified to pH 3 with $HClO_4$. The flow-rate is 2 ml/min, eluent and column temperature 30°C, oxidizing potential +0.8 V vs. an Ag/AgCl reference electrode. All samples were taken through the entire procedure described for urine (see Experimental). The amount of Florisil added was 0.2 g per sample. (A) MHPG-SO_4 without Florisil; (B) urine without Florisil; (C) MHPG-SO_4 with Florisil; (D) urine with Florisil.

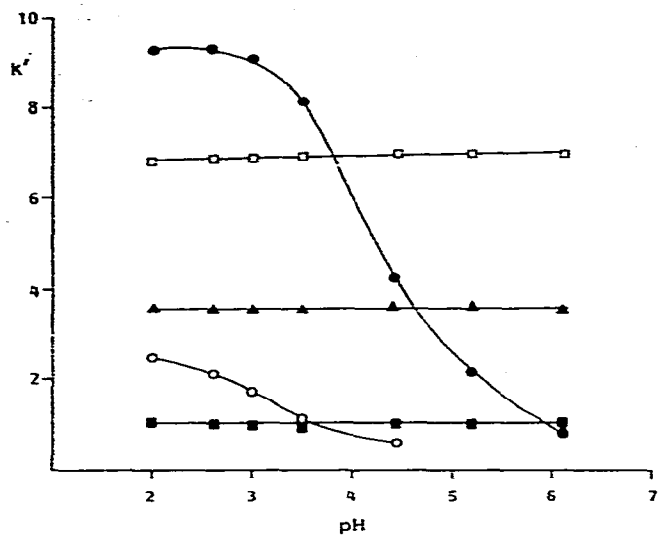


Fig. 4. Retention of MHPG and related compounds as a function of the pH of the mobile phase. (□), DOPET; (▲), MHPG; (■), DHPG; (○), vanillylmandelic acid (VMA); (●), dihydroxyphenylacetic acid (DOPAC). Mobile phase contained 0.5% propanol. See Fig. 3 for further details.

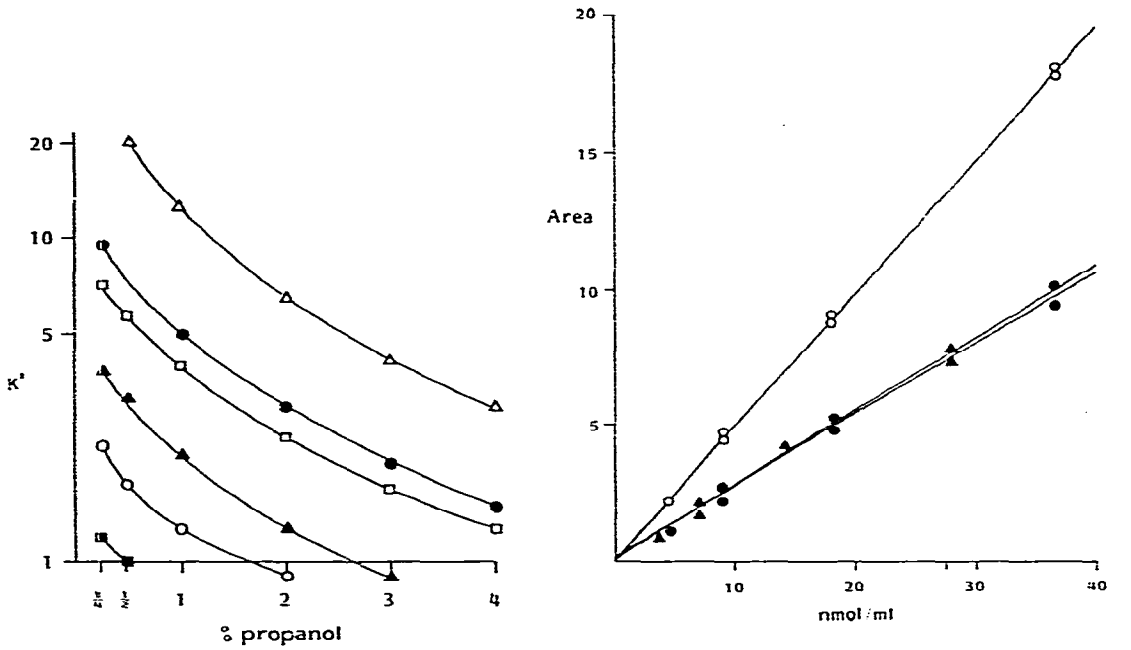


Fig. 5. Retention of MHPG and related compounds as a function of the percentage propanol in the mobile phase. For further details see Fig. 3. (△), MOPET; (□), DOPET; (▲), MHPG; (■), DHPG; (○), VMA; (●), DOPAC.

Fig. 6. Representative calibration curves for MHPG. Detector response as peak area, arbitrary units. (○), Direct chromatography of free MHPG; (●), free MHPG taken through the entire procedure for urine analysis; (▲), MHPG-SO_4 taken through the entire procedure for urine analysis. For further details see Fig. 3

MHPG. Fig. 2 illustrates that extraction of compounds related to MHPG is quite different under the conditions employed for extraction of MHPG from urine. In addition, DHPG and DOPET are unstable at pH 11.5. Finally, Fig. 3 shows that the chromatographic conditions are not very suitable for possible internal standards with retention times different from MHPG. Therefore, the procedure was standardized by running a series of standard solutions through the entire procedure, by adding standards to samples of the urines being analysed and by running the reference urine in all experiments.

Table II summarizes the precision of the assay and recoveries. When values obtained for reference urine were corrected in each experiment for recovery

TABLE II

PRECISION OF THE ASSAY AND RECOVERIES

Values represent coefficient of variation (C.V.) or percentage recovery as mean ± S.D

			Comment
Within-run C.V. (%)	3.1	(n=39)	Variation of duplicate analysis of urine samples of six patients
Day-to-day C.V. (%)	7.9	(n= 5)	Reference urine, when corrected for recovery of standard solutions
Reference urine	16.1	(n= 4)	Reference urine, when corrected for recovery of standard added to urine
Recovery (%)			
Standard solutions	61 ± 7	(n= 6)	MHPG standard
	61 ± 7	(n= 5)	MHPG-SO_4 standard
Standard added to urine	57 ± 8.3	(n =39)	Urine samples of six patients

of the standard added to this urine, a larger coefficient of variation was obtained than when these values were corrected for recovery of the standard solutions in each experiment. Possibly, the latter procedure is more reliable, because eight solutions of standards were used in each experiment, while only one sample of reference urine with added standard was used. This is illustrated by the fact that the variation in recovery of standard solutions is similar to the variation in recovery of standards added to urine, when a sufficiently large number of samples is analysed (Table II). It has been shown that use of an internal standard does not guarantee an improvement of assay precision [12]. In general, Table II shows that the precision of the method is satisfactory. The sensitivity of the method is less than 2.5 nmol/ml, which is sufficient for urine. If necessary, however, the sensitivity can simply be increased by using more urine, and less acetate in the extraction procedure.

Peak identification and interferences

Fig. 7 shows chromatograms of the reference urine, one patient's urine and an MHPG solution with increased chart speed. Retention times of urine peaks at 4.42 and 4.38 min suggests that these peaks represent MHPG. In addition, the similar geometry of the urine peaks compared to that of the MHPG solution suggests that these peaks are without interference.

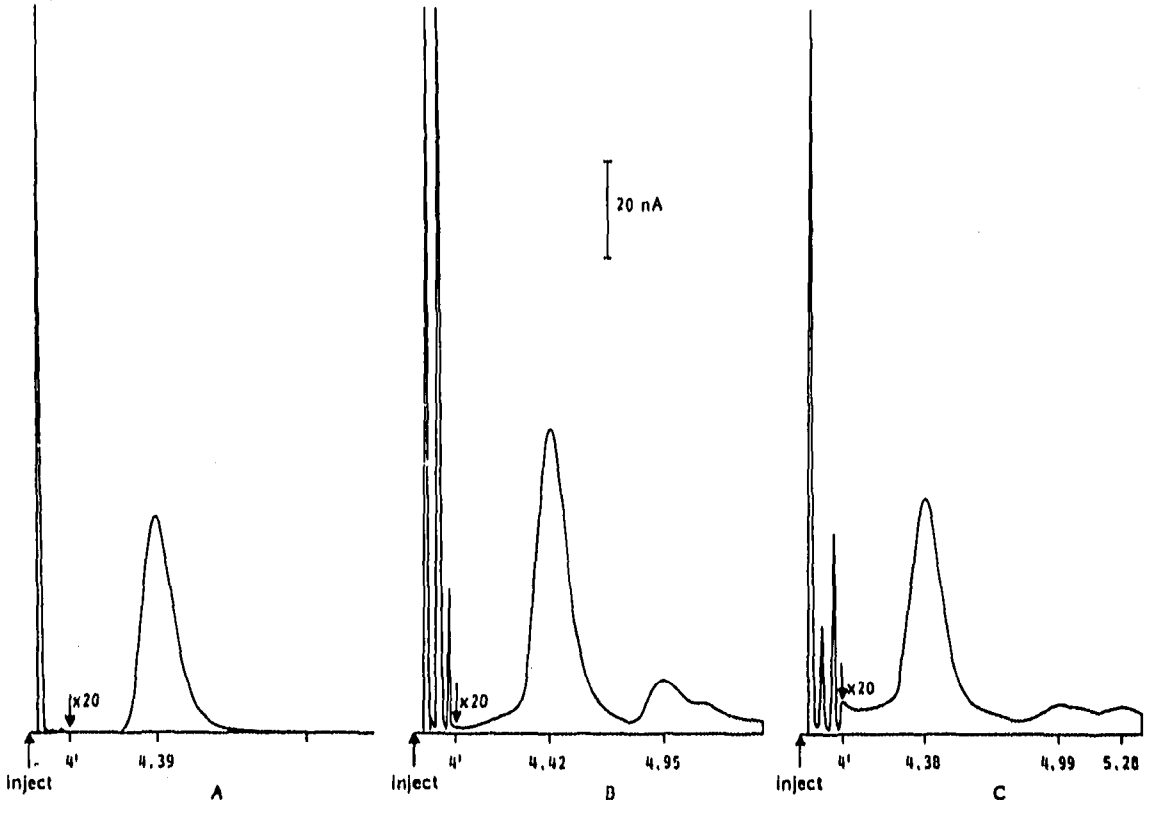


Fig. 7. Chromatograms of MHPG; chart speed 0.25 cm/min, increased 20-fold at 4 min. (A) MHPG reference; (B) reference urine; (C) patient's urine. For further details see Fig. 3.

The MHPG peaks shown in Fig. 7 were also quantified at other oxidizing potentials of the detector. Fig. 8 shows that the assumed MHPG peaks of both urines exhibit characteristics similar to those of the peak of the reference solution. Furthermore, Fig. 9 shows that fractions of the assumed MHPG peak of the reference urine are essentially homogeneous at rechromatography at pH 3 as well as at pH 7. In addition, the total peak area of the assumed MHPG in these fractions was similar at pH 3 and pH 7, suggesting that no interfering compound is present. These tests exclude, in our opinion, the possible presence of a quantitatively important amount of interfering substance in the assumed MHPG peak of the reference urine. Analysis similar to the one shown in Fig. 9 of the other urine sample, shown in Fig. 7, led to a similar conclusion.

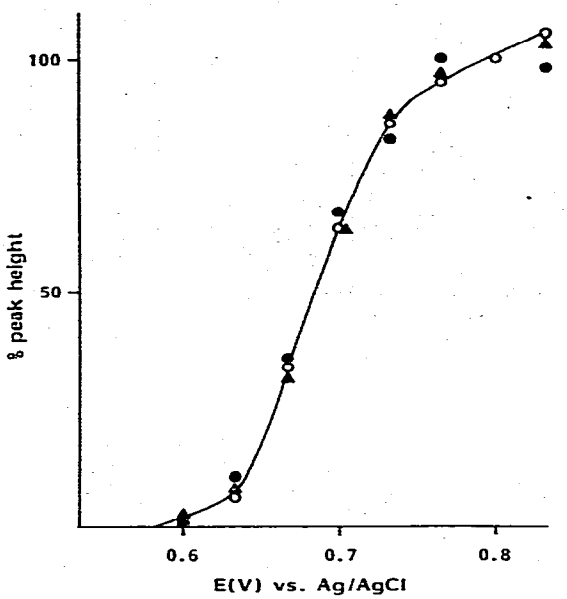


Fig. 8. Relative detector response for assumed MHPG peaks as a function of the oxidizing potential vs. Ag/AgCl reference electrode. (○), MHPG reference (Fig. 7A); (●), reference urine (Fig. 7B); (▲), patient's urine (Fig. 7C). Response at +0.8 V was set at 100% for each sample.

In conclusion, the present method for analysis of urinary MHPG appears to be relatively simple, and reliable. Up to 90 samples can easily be managed in one run. With the present standardization this is equivalent to twenty urine samples. The present method appears to be simpler than gas chromatographic methods [11,13], while reproducibility and selectivity appear to be at least as satisfactory. Existing HPLC procedures for urinary MHPG are more complicated and less reproducible [5], or do not report data for precision [6,7].

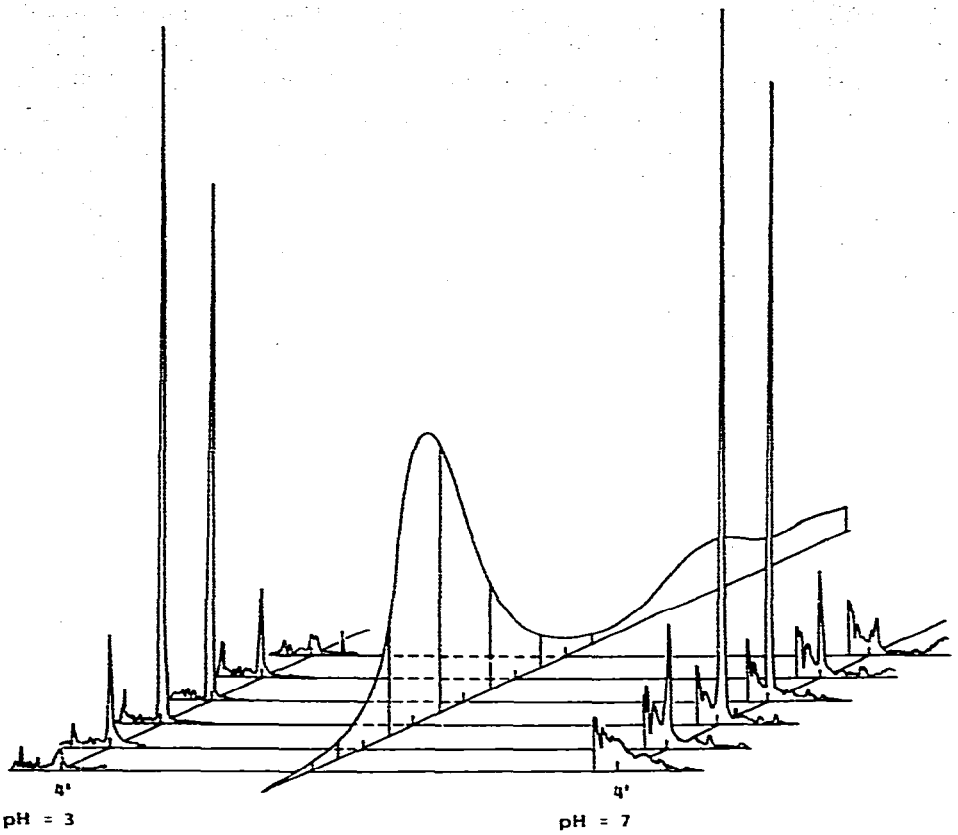


Fig. 9. Rechromatography of fractions of assumed MHPG peak of reference urine (Fig. 7B). Fractions of 200 µl (6 sec) were collected from 4 min on. Centre: original peak of assumed MHPG. Left and right: rechromatography of collected fractions at pH 3 (left) and pH 7 (right). For further details see Figs. 3 and 7.

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