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MEASUREMENT OF URINARY CATECHOLAMINES AND THEIR CATECHOL METABOLITES AND PRECURSOR BY LIQUID CHROMATOGRAPHY WITH COLUMN-SWITCHING AND ON-LINE FLUORIMETRIC AND ELECTROCHEMICAL DETECTION

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SUMMARY

A new method is described for the determination of catecholamines and their precursor (3,4-dihydroxyphenylalanine), and separately of their catechol metabolites (3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethylene glycol and 3,4-dihydroxyphenylethanol) in urine. After a two-step pretreatment involving ethyl acetate extraction and adsorption onto alumina, the separation is performed by ion-pair reversed-phase high-performance liquid chromatography. A column-switching system enables complete separation of the most polar compounds without increase in the total analysis time. The column eluates are monitored with both fluorimetric and amperometric detectors, the relative responses of which are used as an index of peak purity. Reference values for twelve healthy adults are given.

INTRODUCTION

The urinary excretion of catecholamines noradrenaline (NA), adrenaline (A) and dopamine (DA) has been extensively studied since it is postulated to be an index of the peripheral sympathetic nervous activity [1]. In contrast, few methods have been developed for the measurement of the urinary excreted precursor, 3,4-dihydroxyphenylalanine (DOPA), and of metabolites such as 3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylethylene glycol (DOPEG) and 3,4-dihydroxyphenylethanol (DOPET) [2—5].

The estimation of endogenous concentrations of catecholamines and related compounds in biological samples requires highly specific and sensitive procedures. Reversed-phase high-performance liquid chromatography (HPLC)

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with either fluorimetric [6, 7] or electrochemical [8—10] detection has proven to be a useful tool in this regard. However, the methods described are each devoted to the measurement of a small number of catecholamines and/or metabolites.

In order to assess more precisely and completely the metabolic profile of catecholamines in humans, the aim of the present work was the development of an analytical procedure for the determination of NA, A, DA, DOPA and of the catecholamine metabolites DOMA, DOPEG, DOPET and 3,4-dihydroxyphenylacetic acid (DOPAC) using a single urinary sample.

EXPERIMENTAL

Apparatus

The chromatographic analysis was performed with a Model 414 high-pressure pump (Kontron, Zurich, Switzerland) with two pulse dampers in series (an auxiliary high-pressure pump was used to perform the backflush cleaning of columns), an MCS 670 column-switching system (Kontron), equipped with four stainless-steel columns (Fig. 1), a Model SFM 25 spectrofluorimeter (Kontron) with a 10-μl flow cell set at an excitation wavelength of 282 nm and at an emission wavelength of 314 nm (excitation and emission slits 10 and 15 nm, respectively) and a Model LC 4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and an LC-17 glassy carbon transducer. The detector was operated at a setting of +0.72 V versus the Ag/AgCl reference electrode. The system was automated with an MSI 660 autosampler (Kontron) equipped with a 100-μl loop injector and an Anacomp 220 (Kontron) data system for sampling control, flow and column-switching programming, recording and integration. The whole HPLC system was kept in an air-conditioned room (22 ± 1°C).

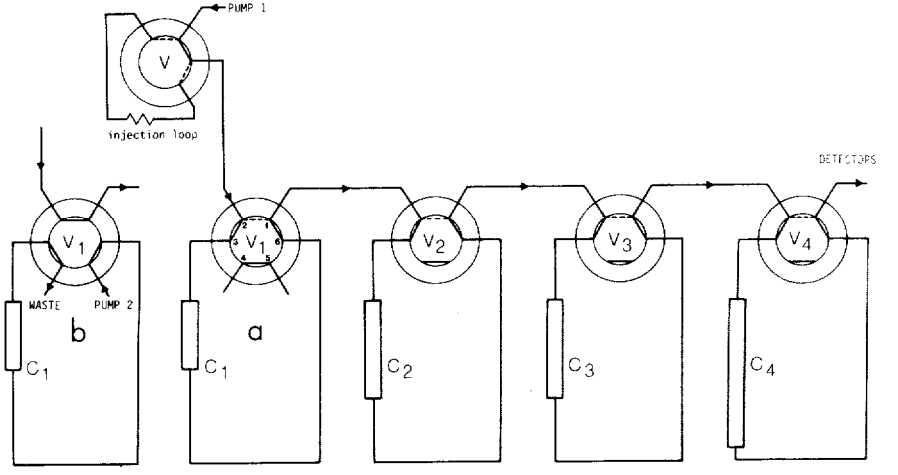

Fig. 1. Configuration of the column-switching apparatus. V = Injection valve; V_1—V_4 = commutation valves; C_1—C_4 = chromatographic columns (I.D. = 4.6 mm) of length 7, 10, 10 and 15 cm, respectively. Parts a and b show the position of valve V_1 for the eluent flow in normal and backflush modes, respectively.

Chemicals

Aluminium oxide was obtained from Serva (Heidelberg, F.R.G.) and prepared according to the method of Anton and Sayre [11]. All other chemicals were of analytical grade and used without further pretreatment.

NA, A, dopamine hydrochloride, DOPA, DOMA, DOPEG, DOPAC and isoproterenol (IP) hydrochloride were purchased from Sigma (St. Louis, MO, U.S.A.); DOPET from Serva and 3,4-dihydroxyphenylpropionic acid (DOPPA) from Aldrich (St. Regis, WI, U.S.A.).

Stock solutions of standards were prepared monthly by dissolving 100 μg/ml of each compound (calculated as the free base for salt-complexed amines) in 0.1 *M* phosphoric acid containing 0.1% of sodium metabisulphite and were stored at 4°C in the dark. Working standard solutions were prepared by diluting the corresponding stock solutions in the same solvent to obtain concentrations between 10 ng/ml and 10 μg/ml.

Chromatographic conditions

The columns were packed with Nucleosil C_{18}, 10 μm (Macherey-Nagel, Düren, F.R.G.), by the ascending slurry-packing technique as described by Bristow et al. [12]. The mobile phase consisted of 0.06 *M* potassium dihydrogen phosphate, 50 mg/l disodium ethylenediaminetetraacetate, 100 mg/l octyl sulphate used as ion-pairing agent and 12.5% methanol. This mixture was adjusted to pH 3.5 ± 0.02 at 22°C with 5 *M* phosphoric acid and degassed in an ultrasonic bath before use. The eluent flow-rate was 0.1 ml/min during the standby periods in order to maintain the stability of columns. It was raised to 0.8 ml/min 1 h before and during chromatographic analysis.

Column-switching system

As shown in Fig. 1, this apparatus was connected to the autosampler injection valve (Model 7010; Rheodyne, Cotati, CA, U.S.A.) and consisted of four valves (Model 7010, Rheodyne) controlled by an electropneumatic unit monitored by an external programmer or an internal keyboard. The four analytical columns (C_1—C_4) were connected across ports 3 and 6 of each corresponding valve (V_1—V_4); their lengths were 7, 10, 10 and 15 cm, respectively. Using the different commutation possibilities, several configurations of the eluent flow could be obtained (Fig. 1).

Extraction procedures

Fresh urine samples were acidified to pH 3 ± 0.2 with 6 *M* hydrochloric acid and then 5-ml aliquots were stored at −20°C until assay (usually within two weeks).

To 1 ml of urine in a 15-ml glass centrifuge tube were added the internal standards IP and DOPPA (each 1 μg) and 5 ml of ethyl acetate. The tube was placed on a rotating shaker (60 rpm) for 10 min and then briefly centrifuged. After transferring the organic phase into another tube, the aqueous phase was re-extracted with 5 ml of ethyl acetate. The organic layers were combined, dried over anhydrous sodium sulphate (ca. 1 g), transferred into a 10-ml conical tube and evaporated to dryness under a stream of nitrogen in a water-bath at 37°C. The residue was reconstituted with 0.6 ml of 0.06 *M* potassium

dihydrogen phosphate—10 g/l disodium ethylenediaminetetraacetate. Therefore, at the end of this step, two samples were obtained: the first one (aqueous phase) contained the catecholamines and DOPA with IP as internal standard; the other one (ethyl acetate extract) contained the acidic and neutral catechol metabolites with DOPPA as internal standard. From each of them, 0.5 ml was transferred into a 1.5-ml Eppendorff tube containing 75 mg of alumina, then 0.3 ml of 1 *M* Tris buffer pH 8.9—20 g/l disodium ethylenediaminetetraacetate was added while vortexing. The tube was placed on an Eppendorff mixer for 15 min, centrifuged and the supernatant was discarded. The alumina was washed with about 10 ml of twice-distilled water according to Todoriki et al. [13] and the catechols were desorbed using 0.8 ml of 0.1 *M* phosphoric acid. The tube was stored at −20°C until chromatography.

Calculations

The endogenous concentrations of catechols in a urine pool were assessed by using the standard addition method (six-point calibration curves). In addition, it was confirmed that these concentrations remained stable for at least two months at −20°C.

During each set of assays of unknown samples (sample), two aliquots of the urine pool were spiked with the highest concentration used for calibration (calibrate) and taken through the entire procedure.

The concentrations in the unknown samples were calculated as follows:

$$\text{Concentration in unknown sample (ng/ml)} = \frac{R_{\text{sample}}}{R_{\text{calibrate}}} \times \text{concentration in spiked urine (ng/ml)}$$

where R is the ratio of the peak height of any compound to that of the internal standard ($R_{\text{calibrate}}$ is the mean of the two determinations) and the concentration in spiked urine is the sum of the endogenous and spiking concentrations.

RESULTS

To achieve the separation of the most rapidly eluted compounds without increasing the total analysis time, the colum-switching technique was found to be of great value. The ability of the system to separate simultaneously the eight catechols and IP from a standard solution has been demonstrated previously [14].

In the case of urine, it appeared that a one-step alumina extraction was insufficient to obtain a valid separation from other endogenous substances. Therefore, a two-step extraction was developed resulting in two samples which have to be chromatographed separately.

Separation of catecholamines and DOPA

Fig. 2a shows the separation of a standard mixture of catecholamines and DOPA together with their internal standard IP. The column-switching system allowed the total analysis time to be reduced from 60 to 35 min by eluting DOPA, NA and A through four columns (length 42 cm), DA through two columns (17 cm) and IP through the first column only (7 cm) according to the switches indicated by the arrows.

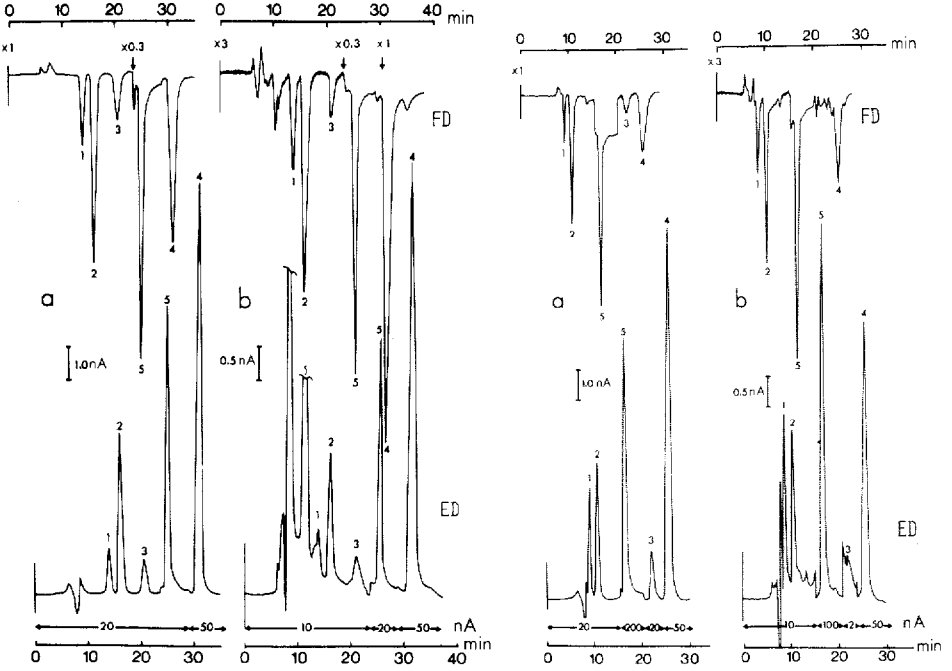

Fig. 2. Chromatograms of a standard solution (a) containing 60 ng/ml DOPA (1), 150 ng/ml NA (2), 45 ng/ml A (3), 750 ng/ml DA (4) and 1000 ng/ml IP (5), and of a human urinary extract (b) after adsorption onto alumina. HPLC conditions as described in Experimental. Injection volume: 100 µl. FD = fluorimetric detection (relative fluorescence); ED = electrochemical detection.

Fig. 3. Chromatograms of a standard solution (a) containing 90 ng/ml DOMA (1), 120 ng/ml DOPEG (2), 45 ng/ml DOPET (3), 1350 ng/ml DOPAC (4) and 1000 ng/ml DOPPA (5), and of a human urinary extract (b) after ethyl acetate extraction and adsorption onto alumina. Other details as in Fig. 2.

A chromatogram of a urine sample is shown in Fig. 2b. Although ethyl acetate extraction resulted in a reduced leading peak and removed some interferences, the peak of DOPA was overlapped in the electrochemical detection (ED) mode, so this compound was only measured in the fluorimetric detection (FD) mode. On the other hand, an electroactive compound frequently coeluted with A making its ED measurement uncertain.

Just after the elution of IP, the column C_1 was backflushed with the mobile phase at 2 ml/min by means of the auxiliary pump while eluting DA from column C_2. Moreover, a rapid cleaning of columns C_2—C_4 at 1.6 ml/min was ensured at the end of the analysis, allowing return to the initial state before the next injection.

Separation of acidic and neutral catechol metabolites

Fig. 3a shows the separation of a standard mixture of DOMA, DOPEG, DOPET and DOPAC together with their internal standard DOPPA. The decrease in the duration of the analysis (from 60 to 30 min) was obtained by eluting DOMA and DOPEG through four columns, DOPET and DOPAC

through three columns and DOPPA through the first column according to the switches indicated by the arrows.

A chromatogram of an extract of urine is shown in Fig. 3b. In that case, all the compounds studied could be detected in both FD and ED modes.

The clean-up of the first column was carried out as described above, i.e., after eluting DOPPA from column C_1 while eluting DOPET and DOPAC from C_3. This step was of particular importance in the case of the ethyl acetate extract which contains several strongly retained compounds. It enabled a considerable saving of time and increased the lifetime of column C_1.

Program monitoring

Before starting the analysis of unknown samples, the switching program was tested by using a standard solution. The within-day reproducibility of the retention times was almost perfect after stabilization of the system, and provided that the ambient temperature was carefully controlled since it markedly influenced the elution pattern.

A slight but continuous decrease in the retention times could be observed over longer periods of time due to the progressive wear of the stationary phase. In order to maintain the column-switching schedule, the first column had to be changed after every 250—300 injections, the second one after every 500—600 injections. The effectiveness of the third and fourth columns remained almost unchanged after 1500 injections.

Linearity and selectivity

For all the compounds the standard addition method was applied to a pooled urine sample so as to check the linearity of the assay. Six aliquots of the pool were spiked with increasing amounts of catechols in the expected range of concentrations to be assayed. The ratios of the peak heights of catechols to that of the corresponding internal standard obtained in both detection modes were plotted against the concentrations added. As shown in Table I, the linear regression coefficients obtained were high and exhibited low standard deviations, demonstrating the linearity of the assay.

TABLE I

REGRESSION LINES OBTAINED BY STANDARD ADDITION OF CATECHOLS TO A HUMAN URINE POOL

n = 6 for all determinations except for electrochemical measurement of A (n = 3).
X-int. is the intercept with the X axis and C.V. its coefficient of variation; N.A. = not available.

Compound	Amount added (ng/ml)	Fluorimetric detection			Electrochemical detection		
		r^2 ± S.D.	X-int. ± S.D. (ng/ml)	C.V. (%)	r^2 ± S.D.	X-int. ± S.D. (ng/ml)	C.V. (%)
DOPA	10 — 60	0.990±0.008	23.4± 1.7	7.1	N.A.	N.A.	N.A.
NA	25 — 150	0.998±0.002	38.6± 3.3	8.7	0.998±0.001	37.6± 3.3	8.6
A	7.5— 45	0.996±0.002	10.3± 0.7	6.3	0.990±0.008	13.4± 1.9	14.1
DA	125 — 750	0.999±0.001	150.8±14.4	9.5	0.999±0.001	143.0±14.4	10.1
DOMA	15 — 90	0.996±0.003	46.8± 4.5	9.5	0.996±0.001	46.4± 5.4	11.6
DOPAC	225 —1350	0.995±0.003	669.6±68.9	10.3	0.999±0.001	605.2±62.9	10.4
DOPEG	20 — 120	0.997±0.002	50.8± 5.4	10.6	0.991±0.013	54.7± 4.6	8.4
DOPET	7.5— 45	0.985±0.014	2.8± 0.8	27.0	0.993±0.006	5.1± 3.0	58.8

The endogenous concentrations in the pool were calculated by extrapolating to zero the peak-height ratios. A close agreement was found between the values obtained with both detection modes for NA, DA, DOMA, DOPEG, DOPET and DOPAC. This can be considered as a valid index of the peak purity. As mentioned above, DOPA and A could not easily be detected by the ED mode, but were clearly free from interferences in FD. For these compounds, the peak purity was assessed by their excitation spectra as described by Krstulovic and Powell [15].

As a consequence, DOPA and A were routinely measured only by FD, NA, DA, DOMA and DOPEG with both detection modes while DOPET and DOPAC were determined only by ED due to their higher response in this mode (see below).

Reproducibility and recoveries

Within-run reproducibility was tested by performing six replicate analyses of a urine sample. Between-run reproducibility was checked by analysing urine samples spiked with several concentrations of catechols over a period of six weeks. The coefficients of variation are given in Table II. For all the compounds, both between-run and within-run coefficients of variation were below 5%, except for DOPET. The within-run reproducibility for DOPET could not be accurately determined due to its low concentration in the urinary sample studied.

The analytical recovery was assessed in the concentration range given in Table II. Overall recoveries averaged 80% for catecholamines, DOPA and IP and 50% for acidic and neutral compounds and DOPPA. No significant difference in recoveries was observed among the compounds of each group over the entire range of concentrations studied.

Minimum detection limits

The sensitivity was calculated as the amount of each compound giving a peak height three times that of the background. Considering the overall ex-

TABLE II

PRECISION AND REPRODUCIBILITY OF CATECHOL MEASUREMENT IN A HUMAN URINE POOL

n = 6 for all determinations. FD and ED are fluorimetric and electrochemical detection, respectively; N.D. = not determined.

Compound	Concentration (ng/ml)	Within-run C.V. (%)		Concentration (ng/ml)	Between-run C.V. (%)	
		FD	ED		FD	ED
DOPA	16	3.7	N.D.	16— 76	5.0	N.D.
NA	35	2.8	2.5	35— 185	3.1	3.0
A	14	4.3	N.D.	14— 59	3.5	N.D.
DA	130	2.5	1.7	130— 880	2.5	2.1
DOMA	36	3.3	3.0	36— 126	3.0	2.8
DOPAC	560	3.6	2.0	560—1910	2.2	1.8
DOPEG	48	2.7	2.9	48— 168	2.3	2.8
DOPET	N.D.	N.D.	N.D.	0— 45	8.3	5.7

TABLE III

SENSITIVITY OF CATECHOL MEASUREMENT IN HUMAN URINE

Compound	Urinary concentrations (ng/ml)	
	FD	ED
DOPA	3.5	N.D.
NA	3.9	1.0
A	4.9	N.D.
DA	5.2	0.6
DOMA	9.6	2.5
DOPAC	95.8	1.6
DOPEG	4.9	2.5
DOPET	10.3	1.0

traction recoveries, the minimum detectable concentrations in urine were far below the concentrations usually encountered in human urine, except for DOPET (see Table III).

Application

Six male and six female healthy volunteers participated in this study (age range: 22—34 years). They were not allowed to ingest drugs, fruit, tyramine-containing and vanillin-rich food the day before and during the study. Urine samples were collected during two consecutive 12-h periods. The results are given in Table IV. As there was no significant difference between men and women for any compound, all the values were pooled. These values are in reasonable agreement with those previously published for DOPA [16], catecholamines [6, 10, 17], DOPAC [9, 18] and slightly lower for DOMA [18, 19]. We were unable to locate any literature data concerning normal urinary excretion of unconjugated DOPEG and DOPET. It should be noted that the concentration of DOPET could be accurately determined in only 9 urine

TABLE IV

MEAN URINARY EXCRETION OF FREE CATECHOLS OVER 12-h PERIODS FOR TWELVE HEALTHY HUMAN SUBJECTS

DOPET values are the mean of four determinations for the period 09—21 h and of five determinations for the period 21—09 h.

Compound	Amount excreted (mean ± S.D.) (μg)	
	09—21 h	21—09 h
DOPA	15.5± 8.0	14.0± 10.2
NA	24.2± 9.5	13.2± 4.3
A	6.6± 4.2	3.1± 1.7
DA	147 ± 42	151 ± 34
DOMA	37.5± 15.9	24.9± 8.0
DOPAC	483 ±144	521 ±115
DOPEG	32.9± 11.2	27.2± 8.3
DOPET	2.6± 1.4	2.8± 1.1

samples out of 24. As might be expected, there was a marked decrease in the amounts of NA and A excreted during the night, and, to a lesser extent, in the amounts of DOMA and DOPEG.

DISCUSSION

The major difficulty in the HPLC analysis of catecholamines and metabolites is related to their chemical heterogeneity which results in large differences in polarity and consequently in retention times. The column-switching technique appears to be a new and fruitful approach to this analytical problem. Hitherto, only one method involving column switching has been published [20], concerning the analysis of catecholamine and serotonin metabolites in cerebrospinal fluid and brain tissue. With regard to catecholamines and their derivatives in urine, the question arises as to whether the sophisticated switching technique offers significant advantages over classical isocratic methods. To our knowledge, there is currently only one published method which describes the HPLC measurement of catecholamines and catechol metabolites in urine [21]. The separation is achieved through three chromatographic steps involving the use of three different mobile phases, the percentage of methanol being increased in two of them during the analysis. In general, there seems to be no possibility to separate the eight catechols within reasonable analysis times by using the same isocratic system. Another technical limitation of such techniques is clearly revealed when the method is applied to complex matrices such as urine. When organic solvents are used in the extraction procedure, some non-polar material is frequently carried over into the extract; then, an extensive and time-consuming clean-up of the column is required between successive injections [22]. The column-switching system allows the immobilization of these late-eluted analytes on the short first column from which they can be rapidly eluted at the end of the analysis or better still during the analysis by means of an auxiliary pump.

In the present work, the eight catechols of interest are divided in two groups with their relative internal standards. It should be noted that a little of the acidic and neutral compounds remains behind in the aqueous phase after the ethyl acetate extraction. However, this is of no consequence since the choice of the internal standards, mobile phase composition and column lengths is such as to avoid interferences between compounds whatever the switching program. Thus, all the studied compounds can be fully separated in a single analysis [14]. When chromatographed separately, due to differences in their waiting times, some compounds from both groups have apparently similar retention times.

A problem common to all HPLC—ED methods is the presence in urine of numerous potentially interfering compounds. Despite the combination of intensive extraction procedures and enhanced separation power, the selectivity is not always assured. The recent development of dual-electrode techniques is very relevant in this regard. The use of two different detectors in series allowed us to quantify some compounds in either detection mode and, in the case of adrenaline, to locate an electroactive interference which otherwise could have led to falsely high values.

Finally, special attention must be given to the versatility of the column-switching system. Given one set of compounds and provided that the separation between them has been obtained, only minor changes in the switching program have to be made for the elution of a particular group of compounds which has been isolated in the course of the extraction.

At the present time, automation of the procedure enables the analysis of 40 urines per day for their content in catecholamines or metabolites. Thus the present method appears suitable for laboratory and clinical studies.

REFERENCES

1 L. Peyrin and Y. Dalmaz, J. Physiol. (Paris), 70 (1975) 353—433.
2 T. Sato and V. de Quattro, J. Lab. Clin. Med., 74 (1969) 672—681.
3 F.A.J. Muskiet, D.C. Fremouw-Ottevangers, J. van der Meulen, B.G. Wolthers and J.A. de Vries, Clin. Chem., 24 (1978) 122—127.
4 F.A.J. Muskiet, D.C. Fremouw-Ottevangers, G.T. Nagel, B.G. Wolthers and J.A. de Vries, Clin. Chem., 24 (1978) 2001—2008.
5 E.B.M. de Jong, B.P.M. Horsten and H.M.J. Goldschmidt, J. Chromatogr., 279 (1983) 563—572.
6 G.P. Jackman, Clin. Chem., 27 (1981) 1202—1204.
7 N.G. Abeling, A.H. van Gennip, H. Overmars and P.A. Voute, Clin. Chim. Acta, 137 (1984) 211—226.
8 R.M. Riggin and P.T. Kissinger, Anal. Chem., 49 (1977) 2109—2111.
9 L.J. Felice, C.S. Bruntlett and P.T. Kissinger, J. Chromatogr., 143 (1977) 407—410.
10 B.H.C. Westerink, F.J. Bosker and J.F. O'Hanlon, Clin. Chem., 28 (1982) 1745—1748.
11 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360—375.
12 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57—64.
13 H. Todoriki, T. Hayashi, H. Naruse and A.Y. Hirakawa, J. Chromatogr., 276 (1983) 45—54.
14 G. Cuisinaud, N. Bernard, C. Julien, C. Rodriguez and J. Sassard, Int. J. Environ. Anal. Chem., 18 (1984) 51—73.
15 A.M. Krstulovic and A.M. Powell, J. Chromatogr., 171 (1979) 345—356.
16 K. Turler and H. Kaser, Clin. Chim. Acta, 32 (1971) 41—51.
17 T.P. Moyer, N.-S. Jiang, G.M. Tyce and S.G. Sheps, Clin. Chem., 25 (1979) 256—263.
18 F.A.J. Muskiet, C.G. Thomasson, A.M. Gerding, D.C. Fremouw-Ottevangers, G.T. Nagel and B.G. Wolthers, Clin. Chem., 25 (1979) 453—460.
19 H. Weil-Malherbe, J. Lab. Clin. Med., 69 (1967) 1025—1034.
20 A. Niederwieser, W. Staudenmann and E. Wetzel, J. Chromatogr., 290 (1984) 237—246.
21 R.D. Hoeldtke and P.L. Stetson, Anal. Biochem., 105 (1980) 207—217.
22 D.D. Koch and G. Polzin, J. Chromatogr., 317 (1984) 327—331.