*Journal of Chromatography*, 420 (1987) 146–151
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3752

## Note

# Determination of 5-S-cysteinyldopa in human urine by direct injection in coupled-column high-performance liquid chromatography

CHRISTER HANSSON

*Department of Dermatology, University of Lund, Lund (Sweden)*

and

BERTIL KÅGEDAL* and MAGNUS KÄLLBERG

*Department of Clinical Chemistry, University Hospital, S-581 85 Linköping (Sweden)*

(First received January 5th, 1987; revised manuscript received April 15th, 1987)

In the normal as well as the malignant melanocyte, pigment formation is initialized by the tyrosinase reaction by which dopaquinone is formed [1]. In the indolic pathway, dopaquinone is further oxidized and transformed into indolic intermediate compounds from which eumelanin is formed. In the phaeomelanin pathway, cysteine or glutathione is added to dopaquinone, which results in a number of dopa-thioethers from which phaeomelanins ultimately are formed.

One of these intermediates, 5-S-cysteinyldopa, has been suggested as a marker of metastazing malignant melanoma [2], and high-performance liquid chromatographic (HPLC) methods with electrochemical detection (ED) have been developed for its determination [3–7].

The clean-up procedures for 5-S-cysteinyldopa from biological samples include purification by adsorption on alumina [3–5], a cation exchanger [5] and a boronic affinity gel [6–8]. Similar sample clean-up procedures have been used for the purification of other catecholamines, such as epinephrine, norepinephrine and dopamine, and already a number of publications have appeared showing the use of (e.g. refs. 9–13) immobilized boric acid.

In boronate affinity chromatography, immobilized boronate forms a cyclic boronate ester with the vicinal *cis*-diols of the catecholamine at high pH. The affinity of various catecholamines for the boronate gel depends on the pH, and the catecholamines can be eluted by decreasing the pH of the eluent. Different catecholic compounds can, therefore, be separated on a boronate affinity column by a pH step gradient [6]. This approach has also been applied to the clean-up of samples before the HPLC quantification of 5-S-cysteinyldopa in urine [7] and serum [8].

0378-4347/87/$03.50 

Some catecholic compounds have also been separated by means of a boronic affinity HPLC column [14]. Without prior purification, the biological sample is injected into the system and the compound of interest is adsorbed on top of the column. By column-switching the compound is eluted and chromatographed. Such methods have been published for determination of epinephrine [15], norepinephrine [15], dopamine [15], dihydroxyphenylalanine [16] and dihydroxyphenylacetic acid [14,16]. Methods for the isolation and detection of 5-S-cysteinyldopa by coupled-column liquid chromatography have been studied in the present work. The affinity properties of this compound on boronic acid silica differ from those of the above-mentioned catecholic compounds, and specific procedures had to be developed.

## EXPERIMENTAL

### *Chemicals*

5-S-L-Cysteinyl-L-dopa was synthesized as described earlier [17,18]. L-Norepinephrine bitartrate and L-tyrosine were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of these compounds were prepared for 5-S-cysteinyldopa in 1 *M* hydrochloric acid, for norepinephrine in 0.1 *M* hydrochloric acid and for tyrosine in water. The mobile phases were 0.1 *M* solutions of phosphoric, formic or acetic acid adjusted to the desired pH with 5 *M* sodium hydroxide. The mobile phases also contained 0.2 m*M* disodium EDTA. For details see figure legends.

### *Chromatography*

Two Constametric III pumps (Laboratory Data Control, Riviera Beach, FL, U.S.A.) were used, together with a Rheodyne sample injector Model 7125 (loop volume 20 or 100 $\mu$l) and a Rheodyne switching valve Model 7010 (Rheodyne, Cotati, CA, U.S.A.). Detection was with a glassy carbon TL-5A electrode, which was operated at +0.75 V vs. an Ag/AgCl (3 *M* sodium chloride) reference electrode RE-1 from Bioanalytical Systems (West Lafayette, NJ, U.S.A.). A potentiostat, LC-2A from Bioanalytical Systems, and a voltmeter, 8020 A Multimeter from Fluke (Tilburg, The Netherlands), were also used. Separations were recorded with a Vitalab 1001 recorder (Vitalab, Maidenhead, Berkshire, U.K.).

The affinity column (100×5.0 mm I.D.) was packed with silica (LiChrosorb, 10 $\mu$m) to which 3-aminobenzene boronic acid was covalently bonded [14]. This packing material, acetylated boronic acid-silica (ABA-silica), is now commercially available from Pierce Sweden (Lund, Sweden). The analytical column (250×4.6 mm I.D.) was packed with Nucleosil $C_{18}$ (7.5 $\mu$m) from Macherey-Nagel (Düren, F.R.G.). Each of the columns was protected by a guard column (150×4.6 mm I.D.) dry-packed with Polygosil $C_{18}$ (40–63 $\mu$m).

### *Urine collection*

Urine samples sent to the laboratory for quantification of 5-S-cysteinyldopa by our routine HPLC–ED method [7] were used in the studies. These urines

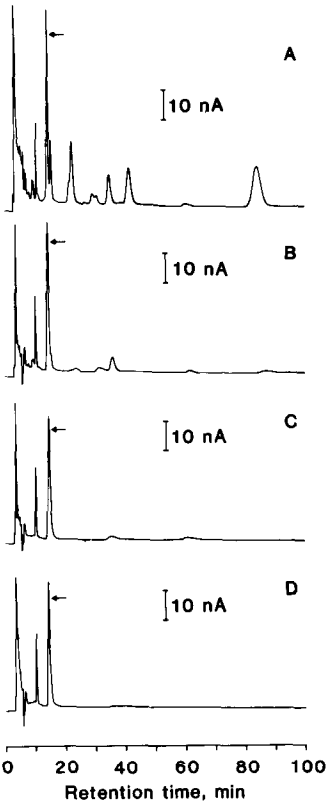


Fig. 1. Urine chromatograms obtained with the coupled-column system. The arrow indicates the 5-S-cysteinyldopa peak. The boronate column was loaded with 100 $\mu$l of urine and then washed with a 0.1 *M* phosphate buffer containing 0.2 m*M* disodium EDTA at a flow-rate of 2 ml/min. Buffer pH and washing time were: (A) pH 7.0 for 5 min; (B) pH 6.0 for 5 min; (C) pH 6.0 for 10 min; (D) pH 6.0 with 10% methanol for 10 min. Chromatography on the analytical column was performed with 0.1 *M* formate containing 0.2 m*M* disodium EDTA (pH 3.0) at a flow-rate of 1 ml/min.

were collected for 24 h in bottles containing thymol–2-propanol as a preservative [7] and stored at −20°C before analysis.

## RESULTS AND DISCUSSION

### *Adsorption on the boronate affinity column*

From earlier studies it was known that the ABA-silica has great affinity for catechols such as dopa, dopamine, epinephrine and norepinephrine at high pH [14]. In the present study we investigated the retention of 5-S-cysteinyldopa on this packing material. Tyrosine, which we used as a control, showed no retention in the pH range 3–6. At pH 6 the capacity factor for 5-S-cysteinyldopa was 100, which was ca. 2.5 times that for norepinephrine. On lowering the pH in the mobile phase we found that the retention of norepinephrine reached zero at pH 4, and that the retention of 5-S-cysteinyldopa was small but still significant at that pH. The results thus showed a very strong affinity for 5-S-cysteinyldopa at pH 6,

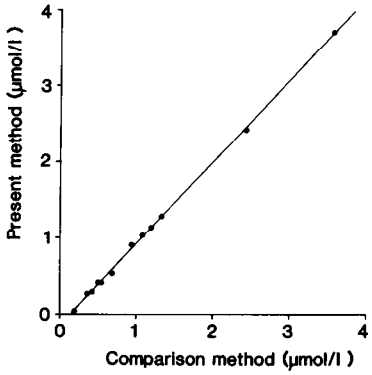


Fig. 2. Comparison of the present method with an earlier HPLC method [7] for determination of 5-S-cysteinyldopa in urine. Regression line: $y = 1.07x - 0.15$ ($r = 0.999$). Urine samples were from patients with malignant melanoma, both with and without metastases. Healthy subjects excrete less than 0.6 μmol of 5-S-cysteinyldopa per 24 h.

which indicates that the compound will be enriched on the top of the column at that pH. These findings were not unexpected, since we earlier found that 5-S-cysteinyldopa could be adsorbed at pH 5 on a boronate gel and prepurified from other electroactive compounds before HPLC [7,8].

The capacity factors both for 5-S-cysteinyldopa and norepinephrine changed very little with addition of methanol to the mobile phase. This could be of importance when other electroactive compounds from biological samples are washed away during clean-up.

*Reversed-phase chromatography of 5-S-cysteinyldopa*

The capacity factor for 5-S-cysteinyldopa on the $C_{18}$ reversed-phase column was also investigated. At pH 2 the capacity factor was 5 and at pH 4 it decreased to 2. These results confirm earlier findings [4] that the retention of cysteinyldopa increases at low pH. The results indicate that the analytical column should be operated at a rather low pH to obtain good separation.

*Purification of urine on the ABA-silica column*

For investigation of the purification of urine on the boronate column, frozen urine samples were thawed and centrifuged. Then 100 μl of clear urine were injected onto the boronate column. After washing, the boronate column was eluted in the backward direction at pH 3, and the eluate was chromatographed by switching the flow into the analytical column.

With a pH of 7 in the first mobile phase, several peaks appeared in addition to the 5-S-cysteinyldopa peak (Fig. 1A). Further purification was obtained at pH 6 (Fig. 1B). Extensive washing further eliminated some of the unrelated components (Fig. 1C), and still better purification was obtained if 10% methanol was added to the mobile phase (Fig. 1D). Slight peak broadening was observed with extensive washing. For analytical purposes we chose 10 min washing with 10% methanol in the mobile phase.

*Comparison with an earlier method*

We had previously developed an HPLC–ED method for determination of 5-S-cysteinyldopa in urine [7]. Particular features of that method are sample pretreatment with a phenylboronate affinity gel and use of the diastereomer 5-S-D-cysteinyl-L-dopa as an internal standard. The method has good precision (coefficient of variation = 6.4%) and analytical recovery is quantitative [7].

Fig. 2 shows the results obtained with the present method compared with those obtained with the earlier method [7]. The close correlation between the results indicates that similar accuracy is obtained. With the present method, the prepurification step is included in the HPLC procedure, and the manually performed clean-up of sample as performed in our earlier method has been omitted. It should be noted, however, that the present method is still manual, and the chromatographic analyses are more time-consuming than in our earlier procedure. The advantage of the new method, however, is that with appropriate equipment the whole procedure can be automated.

## CONCLUSION

We have shown that the boronate affinity HPLC column can be used to purify 5-S-cysteinyldopa from urine before analytical reversed-phase HPLC. This eliminates the manual clean-up procedure before analytical HPLC. The findings indicate that a rapid routine method for the determination of 5-S-cysteinyldopa can be obtained by further automation. In such an automated procedure a shorter boronate affinity HPLC column should be used, and presumably a shorter reversed-phase $C_{18}$ column could be used to shorten the analysis time.

## ACKNOWLEDGEMENTS

This work was supported by the Swedish Cancer Society (Projects No. 2337-B87-01XA and No. 626-B87-15XC), the Medical Society of Linköping (Linköpings Läkaresällskap), the County Council of Östergötland, and the donation funds of the Faculty of Medicine, University of Lund.

## REFERENCES

1 H. Rorsman, G. Agrup, C. Hansson and E. Rosengren, in R.M. MacKie (Editor), Pigment Cell, Vol. 6, Karger, Basel, 1983, pp. 93–115.
2 G. Agrup, P. Agrup, T. Andersson, B. Falck, J.-A. Hansson, S. Jacobsson, H. Rorsman, A.-M. Rosengren and E. Rosengren, Acta Derm. Venereol. (Stockholm), 55 (1975) 337.
3 C. Hansson, L.-E. Edholm, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, Clin. Chim. Acta, 88 (1978) 419.
4 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren, E. Rosengren and L.-E. Edholm, J. Chromatogr., 162 (1979) 7.
5 B. Kågedal and A. Pettersson, J. Chromatogr., 272 (1983) 287.
6 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, J. Chromatogr., 161 (1978) 352.
7 B. Kågedal and A. Pettersson, Clin. Chem., 29 (1983) 2031.

8 B. Kågedal and A. Pettersson, in J. Bagnara, S.N. Klaus, E. Paul and M. Schartl (Editors), Pigment Cell 1985, Biological, Molecular and Clinical Aspects of Pigmentation, University of Tokyo Press, Tokyo, 1985, pp. 721-727.
9 S. Higa, T. Suzuki, A. Hayashi, I. Tsuge and Y. Yamamura, Anal. Biochem., 77 (1977) 18.
10 C.F. Gelijkens and A.P. De Leenheer, J. Chromatogr., 183 (1980) 78.
11 K. Koike, T. Aono, F. Chatani, T. Takemura and K. Kurachi, Life Sci., 30 (1982) 2221.
12 A.J. Speek, J. Odink, J. Schrijver and W.H.P. Schreurs, Clin. Chim. Acta, 128 (1983) 103.
13 T.A. Durkin, E.J. Caliguri, I.N. Mefford, D.M. Lake, I.A. Macdonald, E. Sundström and G. Jonsson, Life Sci., 37 (1985) 1803.
14 L. Hansson, M. Glad and C. Hansson, J. Chromatogr., 265 (1983) 37.
15 P.O. Edlund and D. Westerlund, J. Pharm. Biomed. Anal., 2 (1984) 315.
16 P.O. Edlund, J. Pharm. Biomed. Anal., 4 (1986) 625.
17 G. Agrup, C. Hansson, H. Rorsman, A.-M. Rosengren and E. Rosengren, Communications from the Department of Anatomy, No. 5, University of Lund, Lund, 1976.
18 G. Agrup, L.E. Edholm, H. Rorsman and E. Rosengren, Acta Derm. Venereol. (Stockholm) 63 (1983) 59.