Journal of Chromatography, 344 (1985) 325—331
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2717

Note

High-performance liquid chromatography with fluorimetric detection in biological tissues of the 4-bromomethyl-7-methoxycoumarin ester derivative of 5-pyrrolidone-2-carboxylic acid

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(First received November 27th, 1984; revised manuscript received May 20th, 1985)

5-Pyrrolidone-2-carboxylic acid (PCA) (Fig. 1, 1) is a cyclic derivative of

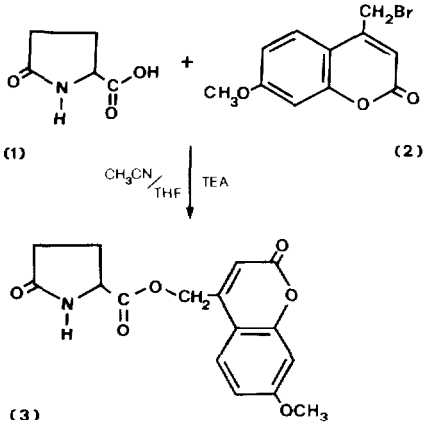

Fig. 1. Reaction of PCA (1) with Br-Mmc (2) to give the fluorescent ester derivative (3).

0378-4347/85/$03.30

glutamic acid and one of the most important compounds associated with the synthesis of glutathione, a universal component of the living cells [1]. PCA occurs both in the free state and as the N-terminal residue of certain mammalian peptides, and its quantitative analysis has received the attention of numerous workers [2–6].

A preceding paper from these laboratories [7] described a simple derivatization method for analysing the PCA distribution in different tissue homogenates by reversed-phase high-performance liquid chromatography (HPLC). Fluorescence detection is more sensitive than UV detection when used in combination with HPLC. Furthermore, chemical modifications of acids by attachment of a fluorescent label facilitate their determination and distribution in various biological materials by HPLC. Esterification is ideally suited for the incorporation of a fluorescent group.

It has been reported [8, 9] that bromomethylcoumarin derivatives react, in nearly quantitative yields, with carboxyl groups to give ester derivatives that allow the fluorescence detection of carboxylic acids in the low picomole range. This method has been used in the analysis of fatty acids by thin-layer chromatography (TLC) [10] and HPLC [11].

With a view to extending the utility of 4-bromomethyl-7-methoxycoumarin (Br-Mmc) (Fig. 1, 2), we investigated the derivatization and analysis of nanomole amounts of PCA in different tissue homogenates from mouse using Br-Mmc. PCA reacts with Br-Mmc in acetonitrile–tetrahydrofuran (2:1, v/v) (triethylamine as base) to give the ester derivative (Fig. 1, 3), which is easily detectable by fluorescence experiments. The procedure is rapid, simple and requires little experience in derivatization techniques.

EXPERIMENTAL

Reagents and chemicals

Br-Mmc was purchased from Fluka (Buchs, Switzerland) and PCA from Ega Chemie (Steinheim, F.R.G.). All organic solvents (Carlo Erba, Milan, Italy) were commercial analytical-reagent grade materials; they were dried with molecular sieves before use and contained no significant fluorescent or absorbing impurities detectable at the highest instrumental sensitivities used. L-[U-^{14}C]Pyroglutamic acid (46.4 MBq/mmol; 1.12 mCi/mmol) was purchased from Amersham, U.K.

Instrumentation

For the quantitative analysis we used: (1) a Varian Model 5010 liquid chromatograph equipped with a 10-μl Valco injector, a Varian Model CDS 111 L integrator, a recorder Model 9176 and a Fluorichrom detector [excitation 7-60 band filter (360 nm), emission 4-76 band filter (530 nm) and 3-72 cut-off filter (440 nm)]; (2) a Kontron System 600 liquid chromatograph equipped with a 20-μl Rheodyne Model 7125 injector and a Kontron Model 200 analytic programmer; detection was performed by a Kontron Model SFM 25 spectrofluorometer (excitation set at 352 nm, emission detected at 410 nm); peak areas and retention times were calculated by a computing integrator Shimadzu Chromatopac C-R 1B. HPLC separations were carried out with a

stainless-steel column (25 × 0.4 cm I.D.) packed with 5-μm Hypersil ODS (Policonsult Scientifica, Rome, Italy). Chromatography was accomplished at room temperature and a pressure of 8.1 MPa. The flow-rate and chart speed were set at 1 ml/min and 0.25 cm/min, respectively. The mobile phase was methanol—70 m*M* phosphoric acid (7:3, v/v).

The fluorescence spectrum of the standard coumarin derivative (Fig. 1, 2) was registered on a Perkin-Elmer Model 650-10 s spectrofluorimeter. Infrared spectra were recorded with a Perkin-Elmer Model 281 spectrophotometer. Mass spectra were run on a JEOL JMS-01 SG-2 mass spectrometer operating with an electron beam energy of 70 eV. Elemental analyses were performed with a Carlo Erba Model 1106 CHN analyser.

Synthesis of PCA ester (standard)

A solution of Br-Mmc (0.25 mmol) in tetrahydrofuran (THF) (1 ml) was gently added to a solution of PCA (0.2 mmol) in acetonitrile (2 ml) containing triethylamine (TEA) (35 μl). The mixture was stirred at 60°C for 30 min. After cooling, the solvent was removed in vacuo to dryness. The residue was collected, washed with cold water, and dried. Recrystallization from water gave a pure sample of PCA-Mmc ester (Fig. 1, 3), m.p. 145—146°C; yield 94%. The PCA-Mmc ester has not been reported before and its structure was confirmed by elemental analysis (calcd. for $C_{16}H_{15}NO_6$: C, 60.55; H. 4.75; N, 4.40; found: C, 60.65; H, 4.70; N, 4.35) and infrared spectroscopy (KBr; ν_{max} 3150, 3045, 2970, 1720, 1615, 1560 and 1515 cm^{-1}) and by mass spectrometry (*m/z* 317, 206, 190, 178, 161, 149, 121 and 84).

Derivatization of PCA in tissue homogenates

Male Swiss albino mice weighing 30 ± 2 g were decapitated and the brain, liver and kidney rapidly removed, weighed and homogenized at 0—4°C in the presence of 5% trichloroacetic acid (TCA) (1:5, w/v). The homogenate was allowed to stand for 30 min at 0°C and then centrifuged at 320 $\bar{g}$ for 10 min. The supernatant fraction was extracted six to eight times with diethyl ether. Aliquots (0.5 ml) of the ether-free aqueous supernatant were dried at 40°C in vacuo. The dried residue was suspended in 5 ml of acetonitrile—THF (2:1, v/v) with 10 μl of TEA and 2 mg of Br-Mmc. The mixture was sealed and heated for 30 min at 60°C in the dark. After cooling, the reaction mixture was filtered into a 10-ml volumetric flask and brought to volume; an aliquot of the resulting solution was chromatographed. Injection volumes were 10 μl.

Recovery study

The recovery of PCA from tissue homogenates was evaluated using L-[U-^{14}C]pyroglutamic acid (46.4 MBq/mmol; 1.12 mCi/mmol). For a statistical validation of data thirty samples of kidney homogenate were used. The amounts of 5.0, 10.0, and 20.0 μg of radioactive PCA (100 000 cpm) (measured with a Packard Tri-Carb Model 4530 instrument) were added to each set of ten samples. The mixtures were extracted as described before. The recovery of radioactive PCA consistently exceeded 90%. These data are summarized in Table I.

TABLE I

RECOVERY STUDY OF PCA ON TEN SAMPLES OF KIDNEY HOMOGENATE

Added amount (μg)	Found amount (mean ± S.D.) (μg)	Coefficient of variation (%)
5.0	4.56 ± 0.12	2.6
10.0	9.19 ± 0.14	1.5
20.0	18.66 ± 0.35	1.9

RESULTS AND DISCUSSION

When PCA was stirred with Br-Mmc, as described under Experimental, a reaction occurred to give the desired fluorescent product (Fig. 1, 1). It was characterized by mass spectrometry and elemental analysis. The mass spectrum showed a molecular ion peak at *m/z* 317 (24%). Significant peaks occurred at *m/z* 206 (100%), 190 (50%), 178 (56%), 161 (38%), 149 (74%), 121 (37%) and 84 (74%), and confirmed the gross structural features. The PCA-Mmc ester is strongly fluorescent and so the development of a sensitive assay method, based on HPLC separation combined with fluorescence detection, is feasible. The ease of derivative formation, coupled with the simple chromatographic separation, indicates the potential of the method for routine analysis of PCA.

Treatment of the biological material as described before was followed by HPLC separation. For identification and quantitative analysis, standard solutions of compounds 2 and 3 were used. The separation of reference mixtures was achieved successfully. Fig. 2 shows the chromatogram of the standards. The peak areas as a function of the amount of PCA-Mmc ester were plotted to determine the reference curve. It was linear over the concentration range 0.8—60 nmol/ml, which indicates that the procedure described here can be used to estimate the concentration of PCA from biological materials (correlation coefficient $r = 0.9997$). The linearity of fluorescence detector response with increasing concentrations of PCA-Mmc ester is expressed as nmol/ml by the relationship $y = 1.06x + 0.133$, where x represents the area ($\times 10^{-6}$) under the peak. The detection limit, based on a peak height versus baseline noise ratio of 10:1, was 30—40 pmol/ml.

The fluorescence quantum yields of carboxylic acids labelled with Br-Mmc are subject to solvent effects [12]. Consequently we have checked the optimum excitation and emission wavelengths of the PCA ester, both in the derivatization mixture and in the separation mobile phase. The uncorrected fluorescence excitation and emission spectra (Fig. 3) of the PCA-Mmc ester were unaltered when measured in the mobile phase rather than the reaction mixture.

The chemical stability of the PCA-Mmc ester is good. Examination of the standard by TLC after several days showed no fluorescent spots other than compound 3. When the PCA-Mmc ester was allowed to stand overnight at room temperature in presence of 5% TCA, the amount of hydrolysed product was 0.1%. No significant differences were found in the derivatization mixture 2—6 h after the sampling.

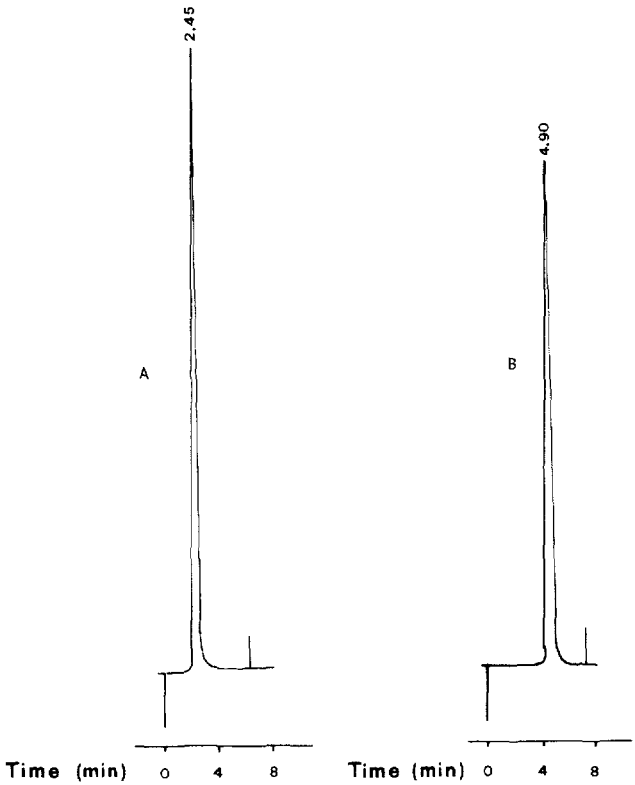

Fig. 2. Chromatography of standards: (A) PCA-Mmc ester; (B) Br-Mmc.

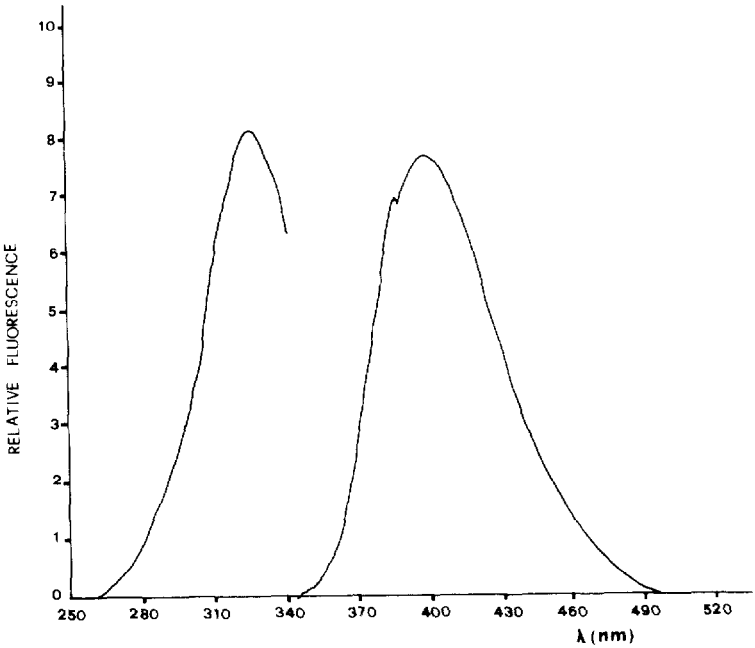

Fig. 3. Uncorrected excitation and emission spectra of PCA-Mmc ester.

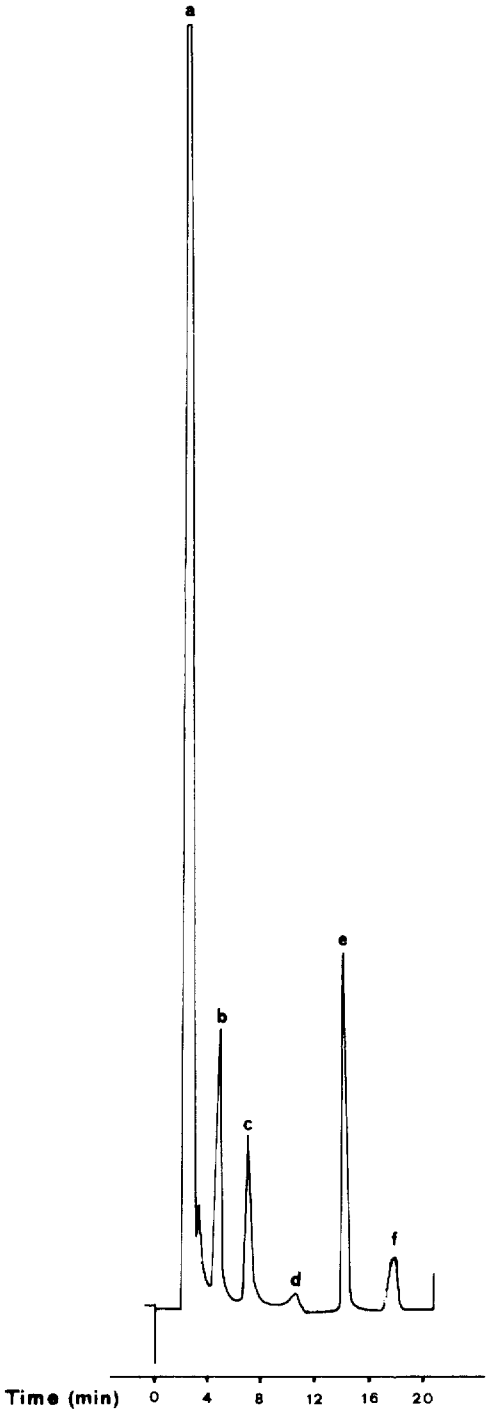

Fig. 4. Chromatography of a derivatization mixture of PCA in liver homogenate. Peaks: a = PCA-Mmc ester (35 nmol/g) (retention time, t_R, 2.45 min); b = Br-Mmc (t_R 4.90 min); c, d, e, and f unidentified.

TABLE II

LEVELS OF PCA IN MOUSE TISSUES

Source	Number of samples	Amount of PCA (nmol/g wet weight of tissue ± S.E.)
Liver	25	29.01 ± 7.85
Kidney	25	62.94 ± 12.6
Brain	25	46.92 ± 10.8

The chromatographic profile of a derivatization mixture in liver homogenate is shown in Fig. 4. The addition of a known amount of PCA-Mmc ester to the sample increases the area and height of the peak at 2.45 min.

The levels of PCA found in liver, kidney and brain of mouse are summarized in Table II and are consistent with those reported in the literature [5].

CONCLUSION

The procedure for the formation of PCA-Mmc ester seems a promising step in the metabolism studies of PCA. The excellent chromatographic and optical properties of the ester derivative, coupled with its chemical stability, allow an accurate separation and evaluation of levels in tissues even in complex biological samples. The method is rapid, highly reproducible and can be applied to routine analysis, as demonstrated by the correlation coefficient and recovery study.

ACKNOWLEDGEMENT

Financial support by Ministero della Pubblica Istruzione (Rome) is gratefully acknowledged.

REFERENCES

1 A. Meister, Science, 130 (1973) 33.
2 N.W. De Lapp and D.K. Dieckman, J. Invest. Dermatol., 68 (1977) 293.
3 R.W. Wolfersberg and J. Tachnick, J. Invest. Dermatol., 62 (1974) 587.
4 P. Polgar and A. Meister, Anal. Biochem., 12 (1965) 338.
5 J. Wilk and M. Orlowski, Anal. Biochem., 69 (1975) 100.
6 R. Dimaline and J.R. Reeve, Jr., J. Chromatogr., 257 (1983) 355.
7 E. Bousquet, V. Guarcello, M.C. Morale and V. Rizza, Anal. Biochem., 131 (1983) 135.
8 J.F. Lawrence, J. Chromatogr. Sci., 17 (1979) 147.
9 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, J. Chromatogr., 234 (1982) 121.
10 W. Dunges, Anal. Chem., 49 (1977) 442.
11 S. Lam and E. Grushka, J. Chromatogr., 158 (1978) 207.
12 J.B.F. Lloyd, J. Chromatogr., 178 (1979) 249.