neous dissolution–remineralization. Further experimental studies are in progress to determine whether the present conclusions regarding the "critical" pK_{FAP} value applies under other conditions (*e.g.*, at other pH values of the remineralizing solution). Also the similarities between the hydroxyapatite pellet experiments and bovine and human teeth studies are being evaluated further.

APPENDIX

Estimation of Calcium-45 Specific Activity at the Bovine Enamel Surface During Simultaneous Demineralization–Remineralization

It has been shown previously (3) that a hydroxyapatite pellet dissolves in the presence of partially saturated buffer containing fluoride when the solution is unsaturated with respect to a pK_{FAP} of ~114. In this dissolution situation, the solution concentration of calcium is higher in the pellet and at the hydroxyapatite surface than in the bulk solution, whereas the fluoride gradient goes the other way, favoring uptake of fluoride from the solution.

In this paper, bovine enamel was studied under similar conditions with the partially saturated bulk solution containing both calcium-45 and fluoride, and the uptake of both species was monitored. If it is assumed that bovine enamel behaves similarly to hydroxyapatite, then when the bulk solution has a pK_{FAP} of >112, the calcium concentration will be higher at the enamel surface since its apparent solubility under these conditions is governed by a pK_{FAP} of ~112. The result is that calcium-45 coming to the surface from the bulk solution will be diluted out by the higher "cold" calcium supplied by hydroxyapatite demineralization. Thus, at sites where calcium and fluoride uptake occurs, it would be expected that measuring uptake of calcium-45 would give too low a value for the calcium uptake as a result of the dilution of calcium-45 by "cold" calcium. The dilution of calcium-45 has been estimated and therefore the expected ^{45}Ca/F uptake ratio (assuming total Ca/F uptake is 10:2) was calculated by using a physical model incorporating the following assumptions:

1. The bovine enamel is assumed to dissolve stoichiometrically.
2. Fluoride and calcium-45 concentrations at the enamel surface are assumed equal to the bulk concentrations.
3. At the enamel surface a suitable expression for describing the surface solution ion activity product is:

$$K_{FAP} = 10^{-110} \text{ or } K_{HAP}(a_{Ca}^{10} a_{PO_4}^{6} a_{OH}^{2}) = 10^{-122}$$

4. The remineralization process in the enamel occurs with the ratio of deposited total Ca/F being 10:2.

Using this model, the expected ^{45}Ca/F uptake ratios have been calculated and seem to agree quite well with the experimental results over a range of bulk solution K_{FAP} values and for solution Ca/P ratios from 1:50 to 50:1.

REFERENCES

(1) M. Yonese, J. L. Fox, N. Nambu, J. J. Hefferren, and W. I. Higuchi, *J. Pharm. Sci.*, **70,** 904 (1981).

(2) W. E. Brown, T. M. Gregory, and L. C. Chow, *Caries Res.*, **11,** 118 (1977).

(3) M. B. Fawzi, Ph.D. Thesis, The University of Michigan, Ann Arbor, Mich. (1976).

(4) E. C. Moreno, T. M. Gregory, and E. W. Brown, *J. Res. Natl. Bur. Std.*, **72A,** 773 (1968).

(5) A. Gee, L. Domingues, and V. Deitz, *Anal. Chem.*, **26,** 1487 (1954).

(6) S. Thiradilok, Ph.D. Thesis, University of Alabama, Birmingham, Ala. (1977).

(7) D. H. Bergstrom, J. L. Fox, and W. I. Higuchi, *J. Pharm. Sci.*, in press.

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Analysis of Monobutyryl and Dibutyryl Derivatives of Adenosine 3′,5′-Monophosphate in Biological Samples Using Isocratic Ion Pair High-Performance Liquid Chromatography

VAL H. SCHAEFFER *, ASAAD N. MASOUD *‡x, and ROBERT J. RUBIN *

Received June 17, 1982, from the **Division of Toxicology, Department of Environmental Health Sciences* and ‡*Department of Anesthesiology/Critical Care Medicine, Johns Hopkins Medical Institutions, Baltimore, MD 21205.* Accepted for publication September 1, 1982.

Abstract □ Adenosine 3′,5′-monophosphate (cyclic AMP), its dibutyryl and monobutyryl derivatives, and a number of other naturally occurring adenine-containing compounds were separated by isocratic ion pair high-performance liquid chromatography. A mobile phase consisting of 30% methanol in 0.1 *M* KH_2PO_4 (pH 4.0) containing 1 m*M* tetramethylammonium hydroxide as the counterion was used to separate the butyryl derivatives. To sufficiently separate cyclic AMP from other adenine-containing compounds, a mobile phase containing 6% methanol in the same aqueous buffer plus counterion was used. Extraction of these cyclic nucleotides from deproteinized biological samples using disposable reverse-phase extraction columns is described. This not only eliminated lipophilic contaminants, but also served to concentrate the samples. The outlined procedures were used to determine the concentrations of the butyryl derivatives in lung tissue and perfusate following a 35-min lung perfusion with 100 μ*M* N^6-$O^{2'}$-dibutyryl cyclic AMP. The role of this technique in the analysis of cyclic nucleotide derivatives as compared with conventional assay procedures is discussed.

Keyphrases □ Adenosine 3′,5′-monophosphate—dibutyryl and monobutyryl derivatives, lung tissue and perfusate, separation by isocratic ion pair high-performance liquid chromatography □ Analogues—dibutyryl and monobutyryl cyclic AMP, lung tissue and perfusate, separation by isocratic ion pair high-performance liquid chromatography □ High-performance liquid chromatography—ion pair, isocratic, cyclic AMP and its dibutyryl and monobutyryl derivatives, lung tissue and perfusate

Analogues of adenosine 3′,5′-monophosphate (cyclic AMP) were first synthesized to selectively mimic the effects of this cyclic nucleotide in various biological systems. The most extensively used analogue has been N^6-$O^{2'}$-dibutyryl cyclic AMP (1). In most instances the dibutyryl derivative has proved to be more biologically active when

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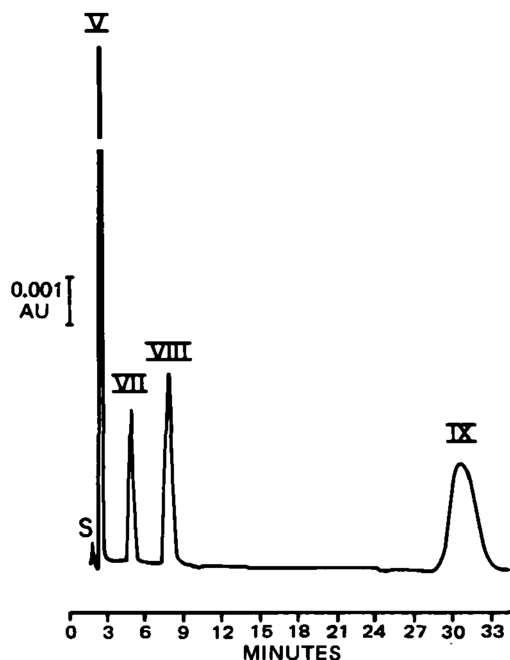

Figure 1—*Chromatogram of a mixture containing reference compounds V (346 pmole), VII (215 pmole), VIII (256 pmole), and IX (1000 pmole). Mobile phase was 30% methanol in 0.1* M KH_2PO_4*–1* mM $(CH_3)_4NOH$. *Other chromatographic conditions are as described under* Experimental.

administered *in vivo* than the unsubstituted cyclic nucleotide, presumably due to its increased lipophilicity (2). It is generally agreed that the dibutyryl cyclic nucleotide is metabolized by most cells to its two monobutyryl derivatives, N^6-monobutyryl cyclic AMP and $O^{2'}$-monobutyryl cyclic AMP (3).

Although the use of these particular cyclic nucleotide analogues has become widespread, the predominant analytical methods for determining the compounds still rely on paper chromatography and TLC (4, 5). These techniques are time consuming and lack the sensitivity and specificity necessary for highly complex mixtures such as biological samples. In recent years, separation by high-performance liquid chromatography (HPLC) has been described (6, 7). The systematic analysis of these compounds in biological extracts, however, has not been reported.

This paper describes the separation of dibutyryl cyclic AMP, its monobutyryl derivatives, cyclic AMP, and several selected adenine nucleotides, nucleosides, and bases most likely to be present in biological samples, using an isocratic ion pair HPLC system. Extraction and quantitation of these compounds from both a rat lung perfusate and lung tissue is also described.

Table I—Mean k' Values for the Four Cyclic Nucleotides Using Three Different Mobile Phases [a]

Compound	k' Mobile Phase A[b]	k' Mobile Phase B[c]	k' Mobile Phase C[d]
V	8.05 ± 0.20	0.33 ± 0.01	0.24 ± 0.01
VII	—[e]	6.79 ± 0.13	1.52 ± 0.02
VIII	—	—	3.07 ± 0.03
IX	—	—	14.97 ± 2.00

[a] k' is defined as $(t_R - t_0)/t_0$ where t_R = retention time of the compound and t_0 = retention time of unretained material (solvent front). k' values are the mean ± *SD* of five determinations representing day-to-day variation. [b] 6% Methanol in 0.1 *M* KH_2PO_4–1 m*M* $(CH_3)_4NOH$ (pH 4). [c] 20% Methanol in 0.1 *M* KH_2PO_4–1 m*M* $(CH_3)_4NOH$ (pH 4). [d] 30% Methanol in 0.1 *M* KH_2PO_4–1 m*M* $(CH_3)_4NOH$ (pH 4). [e] — not eluted.

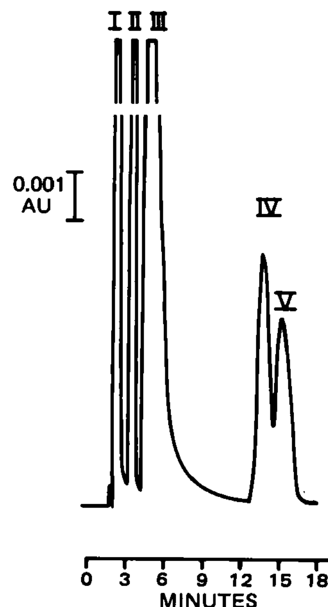

Figure 2—*Chromatogram of a mixture containing reference compounds I (500 pmole), II (1000 pmole), III (1200 pmole), IV (300 pmole), and V (285 pmole). Mobile phase was 6% methanol in 0.1* M KH_2PO_4*–1* mM $(CH_3)_4NOH$. *Other chromatographic conditions are as described under* Experimental.

EXPERIMENTAL

Reagents and Reference Standards—The following compounds were used as chromatographic standards: adenosine 5′-diphosphate sodium salt (I) from equine muscle[1], adenosine 5′-monophosphate sodium crystals[1] (II), assay 99%, adenine[2] (III), adenosine[2] (IV), adenosine 3′,5′-monophosphate sodium crystals[1] (V), theophylline[1] (VI), N^6-monobutyryladenosine 3′,5′-monophosphate sodium salt[1] (VII), $O^{2'}$-monobutyryladenosine 3′,5′-monophosphate sodium salt[1] (VIII), and $N^6,O^{2'}$-dibutyryladenosine 3′,5′-monophosphate sodium salt[1] (IX). All compounds are numbered according to their order of elution.

The following reagents were also used: bovine albumin fraction V[1] (96–99% pure), phosphoric acid[3] (85% pure), tetramethylammonium hydroxide pentahydrate[3], potassium phosphate[3] (reagent grade), 60% perchloric acid[4] (reagent grade), potassium hydroxide[5], and glass-distilled methanol[6]. In-house water was filtered, passed through a reverse osmosis system, glass-distilled, and then demineralized.

Stock solutions of reference standards were prepared at 100 μg/ml in purified water and stored frozen. The stock solutions were diluted to prepare various working standards for chromatography.

HPLC Apparatus and Procedures—The apparatus was similar to that described previously (8). This included an injector equipped with 20-μl loop[7] and a reciprocating pump[8], a 5000-psi gauge, a 1-m pulse damper, a reverse-phase 250 × 4-mm column packed in our laboratory with RP-18[9] (10 μm), and a UV variable-wavelength detector[10].

An aqueous stock solution was prepared containing 1 *M* KH_2PO_4 and 10 m*M* tetramethylammonium hydroxide adjusted to pH 4.0 with phosphoric acid (14.7 *M*). All mobile phases contained a 10-fold dilution of this stock solution, resulting in a final concentration in all mobile phases of 0.1 *M* KH_2PO_4 and 1 m*M* tetramethylammonium hydroxide (pH 4.0). Three mobile phases were used isocratically in this research,

[1] Sigma Chemical Co., St. Louis, Mo. I: grade IX, 97% pure, lot 109C7130; II: 99% pure, lot 87C7170; V: 99% pure, lot 109C7020; VI: lot 16C0135; VII: 97% pure, lot 040F7220; VIII 97% pure, lot 040F7220; IX: 96% pure, lot 100F7340.
[2] National Cancer Institute, Bethesda, Md.
[3] MCB Laboratories, Cincinnati, Ohio.
[4] B&A Laboratories.
[5] Fisher Scientific Co.
[6] Burdick and Jackson, Muskegon, Mich.
[7] Model 7010; Rheodyne.
[8] Model 396; Milton-Roy.
[9] E. Merck Laboratories.
[10] Model Spectro-Monitor III; Laboratory Data Control.

Table II—Cyclic Nucleotide Extraction From Perfusate Using Octadecylsilane Disposable Columns [a]

Compound	Recovery, %	
	30% Methanol	75% Methanol
V	78 ± 2	— [b]
VII	9 + 1	69 ± 4
VIII	—	78 ± 2
IX	—	76 ± 2

[a] Control perfusate was spiked with the compounds. Following deproteinization with 0.6 N perchloric acid and neutralization, supernatant was passed through a C-18 disposable extraction column (1 ml). The column was eluted with 0.5 ml 30% methanol in 0.1 M KH_2PO_4–1 mM $(CH_3)_4NOH$ (pH 4) followed by 0.5 ml of 75% methanol in 0.1 M KH_2PO_4–1 mM $(CH_3)_4NOH$ (pH 4). These 0.5-ml eluates were collected separately and analyzed by HPLC. [b] — not detected.

all of which varied in the methanol concentration: (A) 6% methanol, (B) 20% methanol, and (C) 30% methanol. All mobile phases were filtered through a 0.22-μm membrane filter[11], then deaerated under vacuum prior to use. An aqueous buffer containing 45% methanol was used following chromatography of all tissue extracts to elute strongly retained contaminants. The column was stored in water–methanol (9:1) at the end of each day. The flow rate was 1.2 ml/min., the detector was set at 254 nm with either 0.02 or 0.01 AUFS, and the recorder chart speed was 0.25 cm/min. The void volume, determined by introducing pure methanol on the column, was 1.95 ml.

Preparation of Biological Samples—Perfusate and tissue samples were obtained following perfusion of Krebs–Henseleit buffer supplemented with 4.5% bovine albumin, 0.1 mM dibutyryl derivative (IX), and 0.2 mM theophylline (VI) through a recirculating isolated rat lung preparation for 35 min. After the perfusion, the lungs were immediately frozen in liquid nitrogen, and both the lung tissue and perfusate were stored at −60° until further preparation.

Approximately 1 g of lung tissue was homogenized at 4° with three volumes of 0.6 N perchloric acid. The precipitate was removed following centrifugation at 12,000×g, and the supernatant was neutralized with 10 N KOH. In a similar manner, aliquots of perfusate (0.5 ml) were deproteinized directly by addition of 50 μl of 6 N perchloric acid, and the resulting supernatant fraction was neutralized with 10 N KOH.

The total supernatant fraction was passed through 1-ml octadecylsilane (C-18) disposable extraction columns[12]. These columns were conditioned

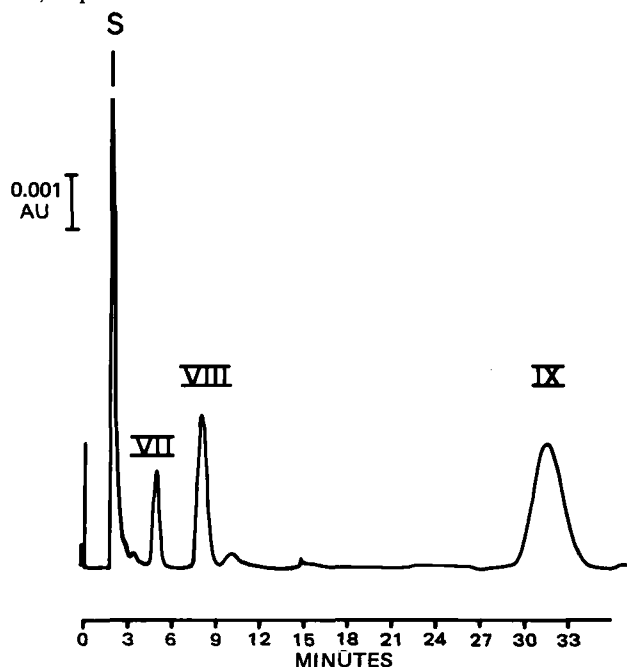

Figure 3—*Krebs buffer supplemented with 100 μM dibutyryl cyclic AMP was subjected to a 35-min sham perfusion, deproteinized in 0.6* N *perchloric acid, and extracted on a C-18 disposable column (see text). Mobile phase used was 30% methanol in 0.1* M *KH_2PO_4–1* mM *$(CH_3)_4NOH$. Other chromatographic conditions are as described under* Experimental. *Key: (S) solvent front.*

[11] Type GS; Millipore Corp.
[12] J. T. Baker, Phillipsburg, N.J.

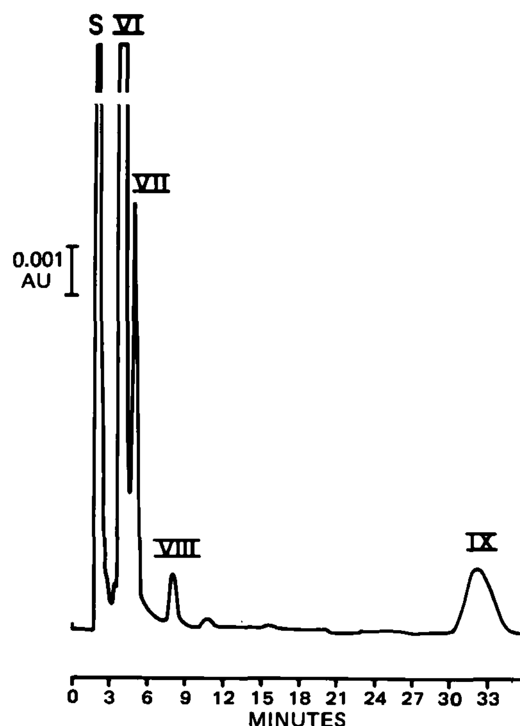

Figure 4—*Krebs buffer supplemented with 100 μM dibutyryl cyclic AMP and 200 μM theophylline was subjected to 35-min lung perfusion, deproteinized in 0.6* N *perchloric acid, and extracted on a C-18 disposable column (see text). Mobile phase was 30% methanol in 0.1* M *KH_2PO_4–1* mM *$(CH_3)_4NOH$. Other chromatographic conditions are as described in* Experimental. *Key: (S) solvent front.*

by the introduction of several volumes of methanol followed by reequilibration with several volumes of the aqueous buffer, 0.1 M KH_2PO_4–1 mM tetramethylammonium hydroxide (pH 4.0). After sample introduction, the columns were first eluted with 0.5 ml of mobile phase C followed by 0.5 ml of 75% methanol in aqueous buffer. Each 0.5-ml fraction was separately collected for HPLC.

RESULTS

Chromatography of Cyclic AMP and Derivatives—Figure 1 illustrates the separation of cyclic AMP and its butyryl derivatives using mobile phase C (30% methanol). The dibutyryl derivative (IX), $O^{2'}$-monobutyryl derivative (VIII), and in most cases the N^6-monobutyryl derivative (VII) were sufficiently retained for analysis in biological samples. However, the unsubstituted cyclic nucleotide (V) eluted too close to the solvent front for adequate separation from the many other nucleotides, nucleosides, and nucleobases eluting at the solvent front and present in biological samples (data not shown). Figure 2 shows the separation of V from four other adenine-containing compounds using mobile phase A (6% methanol). Under these chromatographic conditions, the order of elution is as follows: nucleotide diphosphates, nucleotide monophosphates, nucleobases, nucleosides, and cyclic nucleotides, which possess the longest retention time. Nucleotide triphosphates elute before the diphosphates (data not shown). Since adenosine (IV) is the most retained of the common nucleosides found in biological systems using reverse-phase liquid chromatography (9, 10), all other biological nucleotides, nucleosides, and bases would presumably elute prior to V. Therefore, this procedure theoretically should produce a method suitable for cyclic AMP analysis in biological samples.

The k' values of the four cyclic nucleotide derivatives using three different mobile phases are presented in Table I. Mobile phase A was chosen for determinations of V in biological samples. Both mobile phases B and C were used for the analysis of VII. Compounds VIII and IX were separated using mobile phase C. Standard deviations reflect variation due, for the most part, to different batches of mobile phases, fluctuation in room temperature, and the degree of column equilibration with the mobile phase.

Extraction of Cyclic Nucleotides from Biological Samples—To purify the deproteinized biological material of lipophilic impurities that might shorten the useful lifetime of the chromatographic column, the

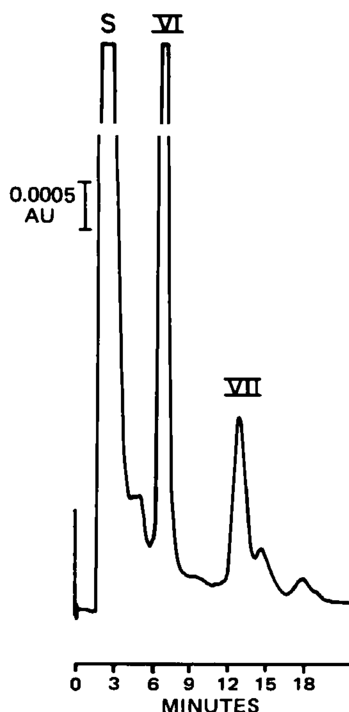

Figure 5—*Lung tissue following 35-min perfusion with 100 μ*M *dibutyryl cyclic AMP and 200 μ*M *theophylline was homogenized, deproteinized with 0.6* N *perchloric acid, and extracted on a C-18 disposable column (see text). Mobile phase was 20% methanol in 0.1* M *KH_2PO_4-1 m*M *$(CH_3)_4NOH$. Other chromatographic conditions are as described under* Experimental. *Key: (S) solvent front.*

Figure 6—*Lung tissue following 35-min perfusion with an unsupplemented Krebs perfusate was homogenized, deproteinized with 0.6* N *perchloric acid, and extracted on a C-18 disposable column (see text). Mobile phase used was 20% methanol in 0.1* M *KH_2PO_4-1 mM $(CH_3)_4NOH$. Other chromatographic conditions are as described under* Experimental.

samples were passed through the C-18 disposable extraction column described earlier. This purification procedure has two additional features that make it extremely attractive. First of all, it served to concentrate cyclic nucleotides by as much as sixfold. Secondly, a separation of V from the butyryl derivatives was accomplished.

Table II shows that 30% methanol in buffer will result in the elution of 78% of V from a lung perfusion medium, while most of VII, VIII, and IX are retained on the extraction column. Application of 75% methanol in buffer will then elute retained cyclic nucleotide analogues (69–78%). Initial elution using <30% methanol produces less recovery of V in the first fraction collected. Percentages of methanol <75% used in the second elution also results in less recovery of the butyryl derivatives. Greater than 75% methanol on the other hand causes increased elution of lipophilic impurities. Thus, the fraction eluted with 30% methanol in buffer was used for the analysis of V, while the butyryl derivatives were analyzed using the fraction eluted with 75% methanol buffer.

Analysis of Cyclic Nucleotides in Biological Samples—A perfusate containing 100 *μM* dibutyryl derivative (no theophylline) was circulated for 35 min in the perfusion apparatus in the absence of a lung. After deproteinization, the perfusate was extracted by the above procedure. The fraction eluted from the column with 30% methanol in buffer was analyzed by HPLC using mobile phase A and was calculated to contain 3.7 ± 1.00 μmoles of V/ml. The HPLC analysis of the 75% methanol fraction from the column using mobile phase C is shown in Fig. 3. It was calculated that each milliliter of the original sham perfusate contained 12.2 ± 0.8 μmoles of VII, 12.0 ± 0.7 μmoles of VIII, and 62.5 ± 2.6 μmoles of IX. Since only small amounts of impurities were found in the commercial supply of IX, these data reflect decomposition during deproteinization.

Figure 4 is a representative chromatogram of a similar perfusate following a 35-min perfusion through a lung. The additional presence of VI (k' = 1.0) should be noted. The perfusate analysis revealed that the concentration of the original dibutyryl derivative had fallen by 55%. Thirteen percent of the initial concentration of IX was recovered as VII. The remaining fraction had presumably entered the intracellular spaces of the lung and been metabolized.

Lung tissue following the 35-min perfusion with or without the dibutyryl derivative was also analyzed by the aforementioned procedures. Chromatographic data using mobile phase C revealed very small amounts of IX (1 nmole/g, wet weight) and VIII (0.5 nmole/g, wet weight) in tissue exposed to IX. The high theophylline content relative to the amounts of VII interfered with the determination of VII in these tissue extracts. To eliminate this problem, mobile phase B was used to analyze VII in lung tissue. Figures 5 and 6 are chromatograms of the tissue perfused with and without the dibutyryl derivative. Compounds VI and VII are well separated under these conditions (Fig. 5). Control tissues do not contain peaks that interfere with either VI or VII (Fig. 6). Following perfusion with 100 *μM* dibutyryl derivative, 10.55 ± 4.87 nmole/g (wet weight) of the monobutyryl derivative (VII) could be recovered from the lung tissue.

Analysis of the unsubstituted cyclic nucleotide (V) in lung tissue exposed to the dibutyryl derivative using mobile phase A was complicated by the fact that control lungs produced a complex chromatogram with one or more peaks coeluting with V. However, extracts of the tissue treated with IX do not consistently differ from unexposed tissue in the region in which V elutes. These results suggest that perfusion with the dibutyryl derivative does not result in significant accumulation of the unsubstituted cyclic nucleotide. However, this can not be established unequivocally until V is separated from the coeluting material.

DISCUSSION

The most common assay techniques for cyclic AMP in biological samples are radioimmunoassay, first described by Steiner *et al.* (11) and modified by Cailla and colleagues (12), and a competitive protein binding assay originally described by Gilman (13) and Brown *et al.* (14). These extremely sensitive methods (<0.1 pmole) appear to be more sensitive than current chromatographic techniques and also eliminate the necessity for complex separations from other nucleotides present in most cellular extracts. Unfortunately, the specificity of these assay systems is no longer adequate when cyclic nucleotide analogues are involved. For example, the affinity of the N^6-butyryl derivative (VII) for the cyclic AMP-dependent binding protein is ~30% of that for cyclic AMP itself (15). The $O^{2'}$-butyryl and dibutyryl derivatives have an even higher affinity than the unsubstituted cyclic nucleotide for the cyclic AMP-specific antibody (12). Therefore, these assay systems are not useful for determining the distribution of the butyryl derivatives.

The methods described here to separate the cyclic nucleotide derivatives do not have the problem of specificity inherent in the more conventional assay systems, since the butyryl groups caused a marked increase in lipophilicity making them easily separable from the parent compound and other nucleotides by HPLC. Although the chromatographic procedure as described here can only detect these compounds to the 0.01–0.1 nmole level, more sensitive UV detection settings, in-

creased amounts injected, and the use of larger extraction columns for trace enrichment of the samples may provide sensitivity comparable to that of the more conventional assays. The separation of cyclic AMP from other biological substances using a simple isocratic HPLC system may be a difficult task. However, the use of gradient elution with a reverse-phase column (10) or the use of ion-exchange chromatography may separate V from the coeluting compound(s) (16).

It should also be pointed out that the chromatogram in Fig. 2 is a good example of the extra dimension offered to the chromatographic process by ion pairing. All compounds analyzed with mobile phase A eluted in the reverse order of their polarity as expected in conventional reverse-phase chromatography. The only exceptions were V and IV. Although V is slightly less hydrophobic than IV, it has a greater retention under the conditions used due to the presence of tetramethylammonium hydroxide. The interaction of the tetramethyammonium cation with the negatively charged V increases its retention. On the other hand, the positively charged IV is not affected by this cation (7). The result is that while IV and V coelute without this cation, its presence causes V to elute after IV so that separation of these two compounds can be achieved.

By the use of the methods described here, it has been shown that the N^6-butyryl derivative is the major product formed following the perfusion of rat lung with dibutyryl cyclic AMP. It is estimated that ~24% of the dibutyryl derivative taken up by the lung during the perfusion period can be recovered as the N^6-monobutyryl analogue from the perfusate and lung compartments. The remaining portion has most likely been further metabolized to the straight-chain monophosphate by the action of phosphodiesterase, since it is not recovered in the lung as the unmetabolized dibutyryl cyclic AMP. We have also shown that 35-min perfusion with 100 μM dibutyryl derivative results in a sevenfold increase in the cyclic AMP content of the lung measured by the protein binding assay of Gilman (13) (data not shown). HPLC analysis reveals that 90% of this increased protein binding can be attributed to the formation of the N^6-butyryl derivative in the lung tissue.

In light of recent findings that different cyclic nucleotide analogues can selectively activate different isozymes of protein kinase (17, 18), it has become important to know the intracellular distribution of these analogues following treatment with cyclic nucleotide derivatives. This analytical procedure provides a specific, sensitive, and simple method for measuring dibutyryl cyclic AMP and its metabolites in biological systems. This technique can potentially be applied for analysis of many of the other cyclic nucleotide analogues. Also, not only can it be used for perfusion studies, but could also be adapted to cell culture and whole animal experiments.

REFERENCES

(1) G. A. Robison, R. W. Butcher, and E. W. Sutherland, *Ann. Rev. Biochem.*, **37**, 149 (1968).
(2) T. Posternak, E. W. Sutherland, and W. F. Henion, *Biochem. Biophys. Acta*, **65**, 558 (1962).
(3) E. Kaukel, K. Mundhenk, and H. Huz, *Eur. J. Biochem.*, **27**, 197 (1972).
(4) E. W. Sutherland and T. W. Rall, *J. Am. Chem. Soc.*, **79**, 3607 (1957).
(5) J. P. Miller, K. H. Boswell, K. Muneyama, L. W. Simon, R. K. Robins, and D. A. Shuman, *Biochemistry*, **12**, 1010 (1973).
(6) J. C. L. V. Luang, N. N. Quang, and G. Hazebroucq, *Adv. Biosci.*, **24**, 201 (1979).
(7) P. J. M. Van Haastert, *J. Chromatogr.*, **210**, 229 (1981).
(8) M. I. Al-Moslih, G. R. Dubes, and A. N. Masoud, *HRC&CC.*, **4**, 173 (1981).
(9) F. S. Anderson and R. C. Murphy, *J. Chromatogr.*, **121**, 251 (1976).
(10) N. E. Hoffman and J. C. Liao, *Anal. Chem.*, **49**, 2231 (1977).
(11) A. L. Steiner, C. W. Parker, and D. M. Kipnis, *J. Biol. Chem.*, **247**, 1106 (1972).
(12) H. L. Cailla, M. S. Racine-Weisbach, and M. A. Delaage, *Anal. Biochem.*, **56**, 394 (1973).
(13) A. G. Gilman, *Proc. Natl. Acad. Sci. USA*, **67**, 305 (1970).
(14) B. L. Brown, J. D. M. Albano, R. P. Ekins, and A. M. Schierzi, *Biochem. J.*, **121**, 561 (1971).
(15) F. A. Nelson and B. M. Birch, *J. Biol. Chem.*, **248**, 8361 (1973).
(16) D. S. Hsu and S. S. Chen, *J. Chromatogr.*, **192**, 193 (1980).
(17) T. S. Yagura, C. C. Sigman, P. A. Sturm, E. J. Reist, H. L. Johnson, and J. P. Miller, *Biochem. Biophys. Res. Commun.*, **92**, 463 (1980).
(18) T. S. Yagura and J. P. Miller, *Biochemistry*, **20**, 879 (1981).

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Subnanogram Quantitation of Chlorpromazine in Plasma by High-Performance Liquid Chromatography with Electrochemical Detection

J. K. COOPER, G. McKAY, and K. K. MIDHA ˣ

Received March 23, 1982, from the *College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, S7N OWO Canada.* Accepted for publication September 17, 1982.

Abstract □ A specific and sensitive high-performance liquid chromatographic (HPLC) method for the quantitative determination of subnanogram levels of chlorpromazine in plasma is described. Following extraction of chlorpromazine and the internal standard, prochlorperazine, HPLC analysis is carried out on a cyano column with a mobile phase consisting of 0.1 M ammonium acetate in acetonitrile (10:90 v/v). The use of oxidative thin-layer amperometric detection allowed the quantitation of 0.25 ng of chlorpromazine/ml of plasma with a coefficient of variation of 5.1%. The HPLC method has adequate sensitivity to follow plasma concentration–time profiles up to 24 hr following low single oral doses of chlorpromazine in healthy volunteers.

Keyphrases □ Chlorpromazine—human plasma, high-performance liquid chromatographic determination of subnanogram levels, electrochemical detection □ High-performance liquid chromatography—electrochemical detection, subnanogram levels, chlorpromazine, human plasma

Chlorpromazine is the most widely used phenothiazine antipsychotic agent. It is extensively metabolized (1–6) both systematically and presystemically (7, 8) to numerous metabolites, some of which are psychoactive. The quantitative analysis of chlorpromazine in plasma or serum has been achieved by several methods which include GLC–

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