High-Pressure Liquid Chromatographic Identification of Drugs Used in Management of Arthritis

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Abstract □ A screening procedure was developed for the identification of drugs used in the clinical treatment of arthritis. Each glucocorticoid, nonsteroidal anti-inflammatory agent, or tranquilizer was characterized by its retention on a reversed-phase high-pressure liquid chromatographic column and by the ratio of the response of dual UV detectors (254 and 280 nm). Although the retention times of all 14 drugs examined were <4 min, each drug could be distinguished easily from the other drugs in the series.

Keyphrases □ Antiarthritis agents—glucocorticoids, nonsteroidal anti-inflammatory agents, tranquilizers, high-pressure liquid chromatographic identification □ High-pressure liquid chromatography—identification of glucocorticoids, nonsteroidal anti-inflammatory agents, and tranquilizers used in arthritis treatment □ Glucocorticoids—identification by high-pressure liquid chromatography, arthritis treatment □ Anti-inflammatory agents, nonsteroidal—identification by high-pressure liquid chromatography, arthritis treatment □ Tranquilizers—identification by high-pressure liquid chromatography, arthritis treatment

Solid dosage forms obtained from patients who received treatment in specialized arthritis clinics in Mexico have been analyzed in these laboratories. In many cases, the patients were told that these drugs were vitamins or natural products and were given a large supply of two or three different tablets to continue taking on their return to the United States. GLC (1) and standard spectroscopic analyses (2) showed that many of these tablets were a tranquilizer (*e.g.*, diazepam or chlordiazepoxide) and an anti-inflammatory steroid or a tranquilizer and a nonsteroidal anti-inflammatory drug (*e.g.*, phenylbutazone or indomethacin). Although there are many qualitative and quantitative analytical methods reported in the literature for individual drugs, a single analytical method for the screening of such samples has not been reported.

BACKGROUND

Since both types of anti-inflammatory agents require different derivatization methods for GLC analysis, high-pressure liquid chromatography (HPLC) was investigated for the identification of the tranquilizers, steroids, and nonsteroidal anti-inflammatory agents.

One major limitation of any chromatographic method for identifying drugs is that many compounds have identical retention times if a fairly large population of compounds is examined. In a recent study (3) of a diverse group of only 101 narcotics, amphetamines, barbiturates, and tranquilizers, only 9% of the drugs could be identified uniquely by HPLC retention times. In that study, if dual UV detectors were used to measure accurately the absorbance of each chromatographic peak at 254 and 280 nm, 95% of the drugs could be characterized uniquely. Moreover, HPLC retention times and A_{254}/A_{280} ratios as paired discriminators were more useful than HPLC retention times on a silica column and on a reversed-phase column as paired discriminators (3). Because of the utility, speed, and low cost of the technique, its usefulness in aiding in the identification of the present group of drugs was investigated.

It is generally recognized that the utility of HPLC retention times is limited because of the small variations that occur with time when the same column is used and because of the large variations that occur with a new column. Relative retention times are considerably more reproducible, but this measurement also has limitations. For example, when reversed-phase HPLC columns are used and the alcohol content of the mobile phase is increased inadvertently or a new column with a lower percent of the stationary phase is used, the observed relative retention time generally increases.

Many of these problems can be reduced or eliminated through the use of a retention index scale based on the relative retention of a homologous series of C_3–C_{23} 2-ketoalkanes and the drug in question (4). The retention index of a given drug is fairly independent of the mobile phase composition, even when the drug retention time varies by several orders of magnitude. The retention index of many drugs also was found to be nearly the same when a bonded cyanoalkyl reversed-phase column was substituted for a C_{18} reversed-phase column (4). Although the retention index measurements were slightly more time consuming than the conventional relative retention time measurements, the former method was selected for this study because of the greater potential for laboratory-to-laboratory reproducibility of the data.

EXPERIMENTAL

Chromatographic Conditions—A 30-cm × 3.9-mm i.d. octadecyl reversed-phase column[1] with a 10-μm particle size was used. The mobile phase was prepared using 3.3 g of dibasic potassium phosphate, 4.2 g of monobasic potassium phosphate, 2.8 liters of methanol, and 1.2 liters of water. The flow rate was 2.0 ml/min, and the column was operated at room temperature.

An HPLC pump[2] and a microsyringe-loaded loop injector[3] also were used. Two UV detectors[4] were used in series (254-nm detector followed by a 280-nm detector), and they were calibrated with a morphine reference standard (3).

Measurement of Retention Indexes—The method used in this study was essentially the same as that reported previously (4). The retention index scale was based on the relative retention of the drug in question and a series (C_3–C_{23}) of 2-ketoalkanes[5]. The capacity factor, k', of the drugs and the standards was determined from the retention time observed for the compound, t_R, and the retention time of the solvent front, t_0, using Eq. 1:

$$k' = \frac{t_R - t_0}{t_0} \qquad \text{(Eq. 1)}$$

The retention index of a given 2-keto alkane standard was, by definition, equal to 100 times the number of carbons in the compound. Thus, 2-butanone was assigned a value of 400. The retention index, I, of a given drug was calculated from the observed capacity factor for the drug, k'_D, the capacity factor for the standard eluting just before the test compound, k'_N, and the capacity factor for the next higher homolog, k'_{N+1}, using Eq. 2:

$$I = 100\,\frac{\log k'_D - \log k'_N}{\log k'_{N+1} - \log k'_N} + 100N \qquad \text{(Eq. 2)}$$

Each drug was chromatographed as a mixture with the 2-ketoalkane reference compounds to minimize the effect of retention time variations on the precision of the retention index measurements. If the chromatographic peak for the drug could not be resolved from the peaks of the standards, the mixture of the standards was chromatographed immediately after the drug. Because of the much smaller molar absorptivity of the 2-ketoalkane standards, a larger quantity of the ketones (~50 μg each) was injected with the drugs (~1 μg) to obtain comparable peak heights.

Preparation of Solid Dosage Forms for HPLC—The tablets were crushed to a fine powder. Approximately 100 mg of the powder was mixed with 1.0–2.0 ml of methanol, and the mixture was then filtered through

[1] μBondapak C_{18} column, Waters Associates, Milford, Mass.
[2] Model M-6000, Waters Associates, Milford, Mass.
[3] Model U6-K, Waters Associates, Milford, Mass.
[4] Model 440, Waters Associates, Milford, Mass.
[5] Analabs Inc., North Haven, Conn.

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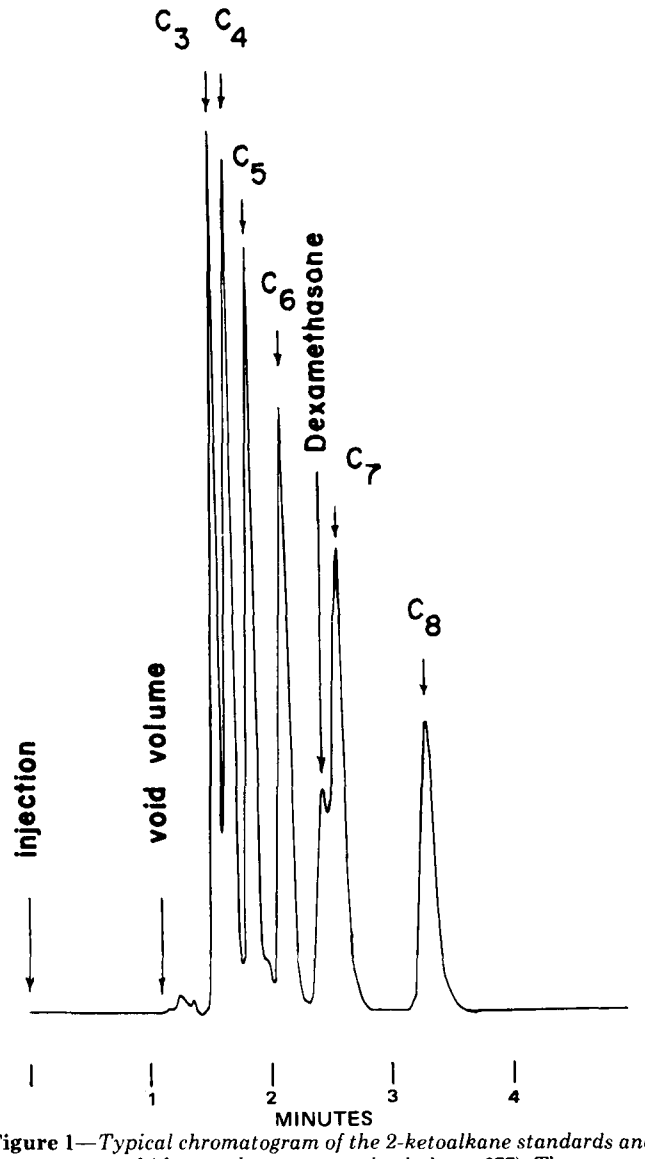

Figure 1—*Typical chromatogram of the 2-ketoalkane standards and a test compound (dexamethasone, retention index = 677). The response of only the 280-nm detector is shown.*

a disposable Pasteur pipet containing a small, tightly packed plug of cotton. Then 0.1–2.0 μl of the methanolic extract was injected into the chromatograph along with the 2-ketoalkane standards mixture.

RESULTS AND DISCUSSION

The chromatogram shown in Fig. 1 for dexamethasone and the 2-ketoalkanes is typical of the results obtained, except that the 254-nm detector response is not shown to simplify the figure. Although the retention times of all of the drugs were fairly short, the drugs were sufficiently removed from the solvent front to obtain precise retention index and absorbance ratio measurements. The longest retention tine was observed for diazepam (3.43 min), the shortest time was observed for naproxen (1.56 min), and the retention time of the solvent front was 1.12 min.

The drugs are listed in Table I in order of increasing retention index, and there is an apparent discrepancy between the retention index and the retention time measurements. Although the short-term reproducibility of the retention time generally was ±0.01 min, the long-term variations were greater. The retention index measurements are considerably more reliable than the retention time measurements (4), and more emphasis was placed on this measurement in the present study.

Although the retention indexes of many drugs in the series were fairly close, each compound could be distinguished from the others if the A_{254}/A_{280} values also were utilized. For example, the retention indexes of fenoprofen and triamcinolone were very close, but the two drugs could be readily distinguished using the absorbance ratio measurements. With

Table I—HPLC Characterization of Various Drugs Used in Management of Arthritis

Drug	Retention Index	A_{254}/A_{280}	Retention Time, min
Naproxen	304	1.61	1.56[a]
Phenylbutazone	363	1.27	1.56
Fenoprofen	439	1.09	1.78
Triamcinolone	463	6.23	1.74
Prednisone	550	6.86	1.97
Ibuprofen	566	6.55	2.03
Mefenamic acid	598	0.51	2.07
Indomethacin	621	1.46	2.16
Prednisolone	625	4.97	2.22
Dexamethasone	677	5.91	2.48
Methylprednisolone	707	5.12	2.64
Chlordiazepoxide	750	1.85	2.91
Methylprednisolone acetate	786	5.06	3.20
Diazepam	812	4.64	3.43

[a] The void volume was 2.24 ml (1.12 min).

methylprednisolone acetate and diazepam, both the retention index and the absorbance ratio measurements were fairly close, but even these two drugs could be differentiated if care was taken in making the measurements. The retention index and absorbance ratio of prednisone and ibuprofen also were grouped closely, but these compounds could be differentiated. The precision of the A_{254}/A_{280} measurements previously was ±1.9% (1), and essentially the same precision was observed in the present study. The short-term precision of the retention index measurements was typically ±5 units. Thus, if the retention index and A_{254}/A_{250} values were used as paired discriminators, all of the drugs in Table I could be distinguished from each other.

All of the steroids had similar absorbance ratio values. The highest value was observed for prednisone (6.86), and the lowest value was observed for prednisolone (4.97). Since all of the steroids in the study had essentially the same chromophore, it was expected that the absorbance ratio also would be similar.

Naproxen, fenoprofen, ibuprofen, mefenamic acid, and indomethacin all contain a carboxylic acid group that is completely ionized at the pH of the mobile phase (7.0). Thus, it was not unexpected that even drugs with a very lipophilic naphthyl group, such as naproxen, had a small retention index. Because of the diversity of the chromophores in this group of drugs, the A_{254}/A_{280} ratios varied greatly.

Although each drug in Table I can be distinguished from the others, other steroids that occasionally were used should have similar indexes and absorbance ratios. The relationship between the drug retention on reversed-phase columns and the lipophilicity of the drug was investigated previously (3, 5–8). Hansch substituent constants, π values, have been used to estimate the lipophilicity of the drugs (9), and these estimates have been used to estimate their HPLC retention indexes (10). The HPLC retention index of a given drug, I_x, could be estimated using the sum of the Hansch substituent values for the drug, π_x, and the experimentally observed index for the reference or parent drug for the series, I_{ref}, by (10):

$$I_x = 200\pi_x + I_{\text{ref}} \qquad \text{(Eq. 3)}$$

With Eq. 3, it is possible to predict other clinically used steroids that might be misidentified as one of the drugs in Table I. It is expected that both the retention index and the absorbance ratio of triamcinolone and meprednisone would be very close. Similarly, there is a potential for interference between dexamethasone (I = 677) and its isomer, betamethasone. Other interferences might be observed between methylprednisolone and triamcinolone diacetate and between methylprednisolone acetate and triamcinolone acetonide.

Other compounds that were not examined in this study also might be misidentified as one of the drugs listed in Table I. Although the absorbance ratio technique greatly reduces the probability of error in the identification of drugs based on retention times alone (3), the methods presented in this study are simple and rapid. As with any analytical method, good laboratory procedures dictate that the initial results should be confirmed by an independent method.

REFERENCES

(1) J. K. Baker, *Anal. Chem.*, **49**, 906 (1977).

(2) E. G. C. Clarke, "Isolation and Identification of Drugs," The

Pharmaceutical Press, London, England, 1969.
(3) J. K. Baker, R. E. Skelton, and C. Y. Ma, *J. Chromatogr.*, **168**, 417 (1979).
(4) J. K. Baker and C. Y. Ma, *ibid.*, **169**, 107 (1979).
(5) J. M. McCall, *J. Med. Chem.*, **18**, 549 (1975).
(6) M. S. Mirrlees, S. J. Moulton, C. T. Murphy, and P. J. Taylor, *ibid.*, **19**, 615 (1976).
(7) D. Henry, J. H. Black, J. L. Anderson, and G. R. Carlson, *ibid.*, **19**, 619 (1976).
(8) K. Miyake and H. Terada, *J. Chromatogr.*, **157**, 386 (1978).
(9) M. S. Tute, *Adv. Drug Res.*, **6**, 1 (1971).
(10) J. K. Baker, *Anal. Chem.*, **51**, 1693 (1979).

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Chemotherapy of Neuroblastoma in Mice with Anticancer Agents

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Abstract □ Neuroblastoma-inoculated A/J mice were treated with various anticancer chemotherapeutic agents, including cyclophosphamide, daunorubicin, vincristine, α-bungarotoxin, dihydroxytryptamine, and diaminopropane. Cyclophosphamide and diaminopropane inhibited neuroblastoma as effectively as bromoacetylcholine and bromoacetate. The effectiveness of these drugs could be related to the inhibition of ornithine decarboxylase, a rate-limiting enzyme for the synthesis of polyamines.

Keyphrases □ Anticancer agents—chemotherapy of neuroblastoma in mice □ Ornithine decarboxylase—inhibition by anticancer agents, chemotherapy of neuroblastoma □ Cyclophosphamide—chemotherapy of neuroblastoma in mice □ Diaminopropane—chemotherapy of neuroblastoma in mice

Despite many efforts, the prognosis of the clinical treatment of neuroblastoma in patients over the age of 1 year is very poor (1). Since the A/J mouse inoculated with C-1300 neuroblastoma cells is considered to be a model of the neuroblastoma disease of humans (2), various anticancer drugs were tested on this animal model to determine an effective way to treat the neuroblastoma disease. Bromoacetylcholine and bromoacetate recently were reported to be effective in inhibiting the neuroblastoma growth *in vivo* (3–6). Therefore, a combination of bromoacetylcholine and other anticancer drugs also was tested in this neuroblastoma mouse model.

METHODS

Adult male A/J mice[1] were inoculated subcutaneously in the interscapular area with 1×10^6 cells of murine C-1300 neuroblastoma cells. Ten days was allowed for a measurable tumor to develop. Drug injections began on the 10th day after neuroblastoma inoculation, and this time was designated as Day 0 of drug treatment. Identical conditions were maintained for the control groups, except that saline was injected instead of drug solutions. Drug treatment was continued for 6 weeks unless otherwise specified. For all injections, the volume of the drug solutions was <50 μl.

Bromoacetylcholine percholate was synthesized by a previously described method (7). Sodium bromoacetate, cyclophosphamide, 1,3-diaminopropane, 5,6-dihydroxytryptamine, α-bungarotoxin, and vincristine were purchased commercially. All drug solutions were freshly prepared at the time of injection.

[1] Jackson Laboratory, Bar Harbor, Me.

RESULTS

It has been established that bromoacetylcholine (30 mg/kg) injected intratumorly one to three times per day and bromoacetate (12 mg/kg) injected intratumorly twice per day inhibit neuroblastoma growth in A/J mice efficiently and prolong the lifespan of these animals by >200% (4, 5). The only anticancer agent that was able to produce an effectiveness equivalent to bromoacetylcholine and bromoacetate was cyclophosphamide (100 mg/kg) injected only once intraperitoneally (Table I). A higher dose (200 mg/kg) of cyclophosphamide reduced its effectiveness due to toxicity. Combination of cyclophosphamide with bromoacetylcholine or bromoacetate did not additionally prolong the lifespan. On the contrary, the lifespan was shortened somewhat as compared to the lifespan of mice receiving the optimal doses of bromoacetylcholine, bromoacetate, and cyclophosphamide administered individually (Table I).

Daunorubicin[2] at a dose of 2 mg/kg injected intravenously twice per week worsened the disease as compared to the control. Combined use of daunorubicin and bromoacetylcholine prolonged the lifespan by only 33%, which was much shorter than the result with bromoacetylcholine alone (Table I).

Vincristine (1 mg/kg) injected intraperitoneally three times daily did not improve the neuroblastoma disease and did not potentiate the effect of bromoacetylcholine (Table I). On the contrary, it shortened the mean lifespan of bromoacetylcholine-treated animals from 73.4 (207% change of lifespan) (5) to 52.1 (129% change of lifespan) days (Table I).

Table II shows the effects of various agents known to inhibit neuroblastoma growth in cell culture on the neuroblastoma growth in A/J mice. α-Bungarotoxin is an inhibitor of neuroblastoma in cell culture (8) but is too toxic to be used *in vivo*. The mice survival rate was shorter than that of the untreated controls (Table II).

5,6-Dihydroxytryptamine has degenerated serotonergic neurons (9, 10) and inhibited gliomas in cell culture (11). However, it did not prolong the lifespan of neuroblastoma-inoculated A/J mice and did not enhance the inhibition of neuroblastoma growth when used in combination with bromoacetylcholine (Table II).

1,3-Diaminopropane was shown to inhibit ornithine decarboxylase, a rate-limiting enzyme for the synthesis of polyamines, which in turn suppressed neuroblastoma growth in cell culture (6). It also produced a significant improvement in mice inoculated with neuroblastoma cells when injected intratumorly twice per day for 6 weeks at a dose of 110 mg/kg (Table II). The combination of diaminopropane and bromoacetylcholine was even more instrumental in prolonging the lifespan of neuroblastoma-inoculated mice.

Various routes of administration were used to find an effective alternative to the intratumor route. Bromoacetylcholine and bromoacetate were ineffective with intraperitoneal administration (Table III), mainly because bromoacetylcholine has a permanent positive charge at the

[2] Obtained from the Drug Liaison and Distribution Section, Division of Cancer Treatment, National Cancer Institute.

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